university of groningen

University Medical Center Groningen

University of Groningen

Uncovering new genes related to heart failure

Lu, Bo

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Lu, B. (2012). *Uncovering new genes related to heart failure*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 06-05-2023

Uncovering New Genes related to Heart Failure

Bo Lu

吕 勃

Financial support by the Groningen University Institute for Drug Exploration (GUIDE) and the University of Groningen for the publication of this thesis is gratefully acknowledged.

Financial support by Greiner Bio-One B.V. for the printing of this thesis is gratefully acknowledged.

Bo Lu is a recipient of Bernouilli Bursary.

Uncovering new genes related to heart failure

©Copyright 2012 Bo Lu
All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, without written permission from the author.

ISBN: 978-90-367-5311-1
ISBN electronic version: 978-90-367-5332-6

Cover design: Bo Lu
Layout: Bo Lu
Printed in: Xinmao Printing Shop, Daqing, China

rijksuniversiteit groningen

Uncovering New Genes related to Heart Failure

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 28 maart 2012
om 16.15 uur

door

Bo Lu

geboren op 19 oktober 1980
te Heilongjiang, China

Promotor:	Prof. dr. W.H. van Gilst
Copromotores:	Dr. H.H.W. Silljé Dr. R.A. de Boer
Beoordelingscommissie:	Prof. dr. M.P. van den Berg Prof. dr. A.A. Voors Prof. dr. J.H. Kingma

Paranimfen: Wardit Tigchelaar
Deli Zhang

To my family

献给我的家人

Contents

Chapter 1

Introduction and aim of the thesis

Introduction

Chronic heart failure (CHF) is the common end-stage entity of many cardiovascular diseases. It is defined as the inability of the heart to supply adequate blood in response to systemic demands. Clinical symptoms include decreased exercise capacity, shortness of breath, fatigue and edema. The incidence of CHF is 2-3% in developed countries, and rises sharply to 10% in those aged 75 years and older [1]. Treatment of CHF has improved over the past decades, by treatment options including angiotensin converting enzyme inhibitors, beta-blockers, aldosterone antagonists and angiotensin receptor blockers. Despite this success, CHF is still characterized by significant mortality with a 5-year mortality rate of approximately 50% [2, 3].

Cardiac remodeling

Ischemic heart disease, hypertension, valve disease, myocarditis and cardiomyopathy are the main causes of CHF development [1]. All of these pathologic conditions lead to a series of molecular, cellular and interstitial changes in the heart termed as cardiac remodeling [4]. Cardiomyocyte hypertrophy (growth) is one of the main remodeling processes triggered by biomechanical stress on the heart and/or neurohumoral changes (Figure 1) [5]. This is accompanied with enhanced sarcomere organization and re-expression of fetal genes such as atrial natriuretic peptide (ANP) [6]. This is considered to be an adaptive response to keep cardiac output within the normal range and to decrease ventricular wall stress. In the short term, this is believed to be a beneficial compensatory mechanism (compensated stage), but in the long run these processes can lead to CHF (decompensated stage) [7]. In addition to growth of cardiomyocytes, rearrangement of cardiac muscle fibers, and accumulation of extracellular matrix components, fibrosis, occur during pathological hypertrophy [7]. The latter processes are nearly absent in physiological hypertrophy, caused by exercise or pregnancy (Figure 1) [8]. It has been suggested that it is the nature of the stimulus, not whether it is intermittent or sustained, that determines the kind of hypertrophy [9]. So far, however, the molecular mechanisms are still poorly understood and a comprehensive view on the genes involved is still lacking.

Altered expression pattern in CHF

Alterations in gene expression have been described in cardiac remodeling and CHF. These changes in gene expression patterns often reflect the fetal gene expression patterns and it is

generally believed that in CHF cardiac expression returns to the fetal gene program. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are probably the best documented members of the fetal gene program. ANP is predominantly synthesized in the atria, while BNP is mainly expressed in the ventricles of the adult heart [10]. These extracellular proteins are secreted by the heart with a strong elevation during HF [11]. These peptide hormones can therefore be used as plasma markers for HF and because of its stability the NT- proBNP form is generally used as a clinical HF marker. Upon their release, ANP and BNP show diuretic, natriuretic and vasorelaxant effects. Besides this, ANP and BNP also act as paracrine factors,exerting antihypertrophic and antifibrotic effects in the heart [12]. Not all gene expression changes during HF development appear, however, to be beneficial. The cardiac isoform of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2a) is a calcium ion (Ca^{2+}) pump powered by ATP hydrolysis. SERCA2a has an important role in cardiomyocyte Ca^{2+} regulation [13] and in HF, SERCA2a expression is significantly decreased, which leads to poor Ca^{2+} handling and a deficient contractile function [14, 15]. Although it is still debated whether return to the fetal gene program is in general beneficial or detrimental, the examples above show that per gene the effects on HF development and progression have to be determined. This will require specialized and genetically tractable HF models.

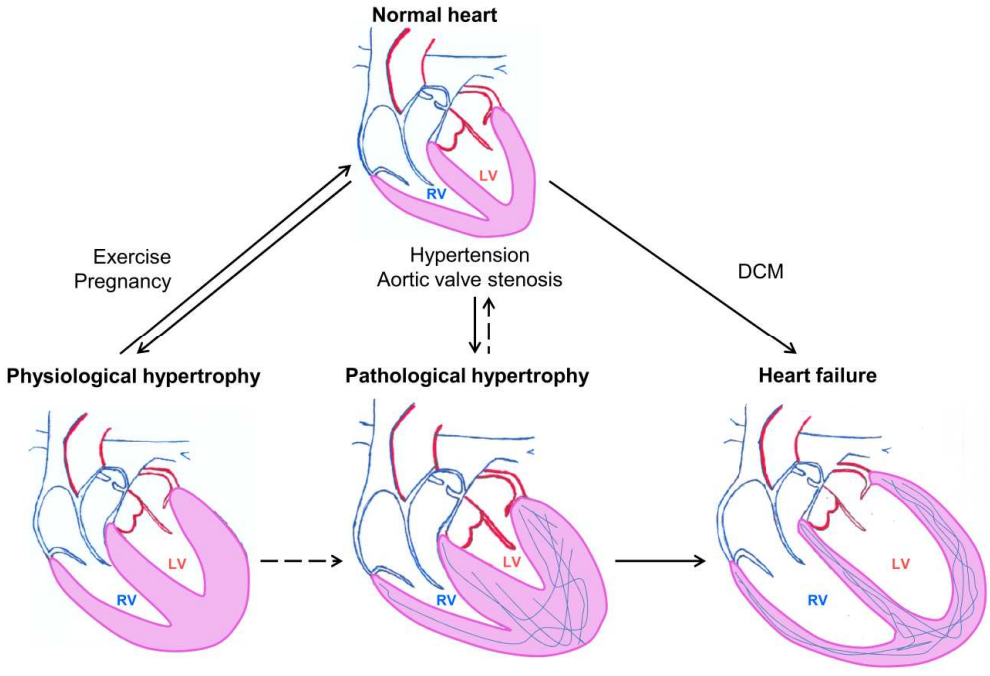


Figure 1. Formation of different types of cardiac hypertrophy

In vivo and *in vitro* models to study heart failure

During the past decades, several models have been developed to investigate molecular mechanisms, pathology, progression, complication and treatments of CHF. Among them, animal models, especially rodent models, remain the most popular tool for providing insights into CHF. Multiple intervention techniques have been developed to generate HF in rodents [16], as outlined in Table 1. Moreover, rodents offer many advantages such as small size, low cost, easy housing and care and most important genetic manipulation of mice and to a lesser extent also rats is relatively easy. As 99% of the human genes have orthologs in mice, gene targeting technology allows generation of transgenic mice to mimic patient situations [17] and to investigate cardiac specific functions of particular genes.

Table 1. Rodent models of heart failure

Aetiology	Techniques	Advantages	Limitations
Pressure overload	Aortic banding	Valuable to study hypertrophy and transition to heart failure.	Often large variations in cardiac hypertrophy development between animals.
Volume overload	Aortocaval shunt	No thoractomy, simple and relatively easy technique.	Rather a model of circulatory failure than heart failure.
Myocardial ischemia	Coronary artery ligation	Difficult and rather high drop-out rates.	Variation of infarct size, local insult to otherwise normal heart.
Neurohormonal induced cardiac hypertrophy	Insertion of osmotic minipumps with neurohormones	Easy to perform and reproducible.	Somewhat artificial infusion of high doses of neurohormones.
Systemic hypertension	Ren2; SHR; SHHF; Dahl salt sensitive rats	Non-invasive, does not require intervention for disease induction.	Heart failure develops already at a young age.
Genetic modifications	Transgenesis or knock out	Allows to study the cardiac role of one particular gene.	Often extreme conditions. E.g. no expression at all or very high overexpression.

Besides *in vivo* animal models, cultured primary rat neonatal cardiomyocytes have been widely used to study cardiomyocyte hypertrophy *in vitro*. A number of extracellular stimuli, including endothelin-1 (ET-1), isoproterenol, transforming growth factor-β, tumor necrosis factor-α, insulin-like growth factor-I, and angiotensin II (Ang II) can individually induce hypertrophy in these *in vitro* cultured cardiomyocytes [18-22]. However, since CHF is multifactorial syndrome and genetic, systemic and environmental factors play important roles,

there are no perfect models that can reproduce completely the human situation. Therefore, depending on the research question multiple models might be required to answer specific problems.

Scope of this thesis

As outlined above, cardiac remodelling is accompanied by changes in gene expression and these changes could have beneficial or detrimental roles in CHF development. In this thesis we searched for novel genes with altered gene expression profiles in multiple HF and hypertrophy models. This resulted, amongst others, in the identification of a novel gene, termed Dhrs7c, which is downregulated during heart failure. An initial characterization of Dhrs7c was performed and a transgenic animal model was generated to explore its potential role in heart failure development.

In **Chapter 2**, we review gene expression changes in heart failure and the process of cardiac remodeling. In **Chapter 3**, we describe the isolation and culturing of primary neonatal cardiomyocytes and tested whether an anti-mitotic drug that inhibits Plk1activity could improve purity of this culture by blocking fibroblast proliferation. **Chapter 4** describes how we identified genes associated with HF/hypertrophy development by combining gene expression data obtained from *in vitro* and *in vivo* experiments. In **Chapter 5**, a basic characterization of one of the identified genes, termed Dhrs7c, is presented. In **Chapter 6**, the generation and characterization of a transgenic mouse with cardiac specific over-expression of Dhrs7c is described. Finally, in **Chapter 7** we will discuss the findings described in the previous chapters.

References

1. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, Stromberg A, van Veldhuisen DJ, Atar D, Hoes AW, Keren A, Mebazaa A, Nieminen M, Priori SG, Swedberg K, ESC Committee for Practice Guidelines (CPG). ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: The task force for the diagnosis and treatment of acute and chronic heart failure 2008 of the european society of cardiology. developed in collaboration with the heart failure association of the ESC (HFA) and endorsed by the european society of intensive care medicine (ESICM). *Eur J Heart Fail*. 2008;10:933-989.

2. Stewart S, MacIntyre K, Hole DJ, Capewell S, McMurray JJ. More 'malignant' than cancer? five-year survival following a first admission for heart failure. *Eur J Heart Fail*. 2001;3:315-322.

3. Ho KK, Anderson KM, Kannel WB, Grossman W, Levy D. Survival after the onset of congestive heart failure in framingham heart study subjects. *Circulation*. 1993;88:107-115.

4. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. experimental observations and clinical implications. *Circulation*. 1990;81:1161-1172.

5. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7:589-600.

6. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: A strong hypothesis. *Heart Fail Rev*. 2007;12:331-343.

7. Hill JA, Olson EN. Cardiac plasticity. *N Engl J Med*. 2008;358:1370-1380.

8. Dorn GW,2nd. The fuzzy logic of physiological cardiac hypertrophy. *Hypertension*. 2007;49:962-970.

9. Perrino C, Naga Prasad SV, Mao L, Noma T, Yan Z, Kim HS, Smithies O, Rockman HA. Intermittent pressure overload triggers hypertrophy-independent cardiac dysfunction and vascular rarefaction. *J Clin Invest*. 2006;116:1547-1560.

10. Nobata S, Ventura A, Kaiya H, Takei Y. Diversified cardiovascular actions of six homologous natriuretic peptides (ANP, BNP, VNP, CNP1, CNP3, and CNP4) in conscious eels. *Am J Physiol Regul Integr Comp Physiol*. 2010;298:R1549-59.

11. Mukoyama M, Nakao K, Hosoda K, Suga S, Saito Y, Ogawa Y, Shirakami G, Jougasaki M, Obata K, Yasue H. Brain natriuretic peptide as a novel cardiac hormone in humans. evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. *J Clin Invest*. 1991;87:1402-1412.

12. Nakao K, Itoh H, Saito Y, Mukoyama M, Ogawa Y. The natriuretic peptide family. *Curr Opin Nephrol Hypertens*. 1996;5:4-11.

13. Periasamy M, Bhupathy P, Babu GJ. Regulation of sarcoplasmic reticulum Ca2+ ATPase pump expression and its relevance to cardiac muscle physiology and pathology. *Cardiovasc Res*. 2008;77:265-273.

14. Meyer M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. *Circulation*. 1995;92:778-784.

15. Schwinger RH, Bohm M, Schmidt U, Karczewski P, Bavendiek U, Flesch M, Krause EG, Erdmann E. Unchanged protein levels of SERCA II and phospholamban but reduced Ca2+ uptake and ca(2+)-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. *Circulation*. 1995;92:3220-3228.

16. Balakumar P, Singh AP, Singh M. Rodent models of heart failure. *J Pharmacol Toxicol Methods*. 2007;56:1-10.

17. Ruiz P, Witt H. Microarray analysis to evaluate different animal models for human heart failure. *J Mol Cell Cardiol*. 2006;40:13-15.

18. Sadoshima J, Izumo S. Signal transduction pathways of angiotensin II--induced c-fos gene expression in cardiac myocytes in vitro. roles of phospholipid-derived second messengers. *Circ Res*. 1993;73:424-438.

19. Ito H, Hiroe M, Hirata Y, Tsujino M, Adachi S, Shichiri M, Koike A, Nogami A, Marumo F. Insulin-like growth factor-I induces hypertrophy with enhanced expression of muscle specific genes in cultured rat cardiomyocytes. *Circulation*. 1993;87:1715-1721.

20. Asakawa M, Takano H, Nagai T, Uozumi H, Hasegawa H, Kubota N, Saito T, Masuda Y, Kadowaki T, Komuro I. Peroxisome proliferator-activated receptor gamma plays a critical role in inhibition of cardiac hypertrophy in vitro and in vivo. *Circulation*. 2002;105:1240-1246.

21. Liao Y, Asakura M, Takashima S, Ogai A, Asano Y, Shintani Y, Minamino T, Asanuma H, Sanada S, Kim J, Kitamura S, Tomoike H, Hori M, Kitakaze M. Celiprolol, a vasodilatory beta-blocker, inhibits pressure overload-induced cardiac hypertrophy and prevents the transition to heart failure via nitric oxide-dependent mechanisms in mice. *Circulation*. 2004;110:692-699.

22. Irukayama-Tomobe Y, Miyauchi T, Sakai S, Kasuya Y, Ogata T, Takanashi M, Iemitsu M, Sudo T, Goto K, Yamaguchi I. Endothelin-1-induced cardiac hypertrophy is inhibited by activation of peroxisome proliferator-activated receptor-alpha partly via blockade of c-jun NH2-terminal kinase pathway. *Circulation*. 2004;109:904-910.

Chapter 2

Hypertrophy and fibrosis in heart failure

Introduction

Heart failure (HF) is characterized by reduced cardiac output and an increased venous return. HF is often the common final entity of many cardiovascular diseases, such as pressure overload (hypertension), volume overload (mitral regurgitation), myocardial infarction, myocarditis and cardiomyopathy. Despite these diverse aetiologies, HF patients share many common features. Clinically, HF patients have often decreased exercise capacity, shortness of breath, fatigue and edema. Prognosis for HF is still poor with a mortality rate of approximately 50% within 5 years [1]. HF is a chronic disease and with the ageing population the incidence of HF will increase and hence the burdens on our health care systems [2].

2.1. Cardiac remodeling

Cardiac remodeling mostly refers to ventricular remodeling and can be defined as any structural and functional change of the heart before and during HF, such as heart size, shape and mass [3]. In the beginning, it is a compensatory reaction of the heart to reduce stress on the ventricular wall in response to changes in afterload (pressure load), preload (volume load) or myocardial injury [4]. However, continued remodeling may not be necessary to maintain the integrity of the circulation [4]. Progressive remodeling is considered as an adverse event that leads to development and progression of HF [3, 5].

One of the major features of cardiac remodeling is cardiac hypertrophy, which refers to the increase of heart mass and/or size. Cardiac hypertrophy can be triggered by a combined action of mechanical stress and humoral trophic factors resulting in cardiomyocyte growth [6, 7]. It is generally believed to be an adaptive and protective mechanism since it also occurs in athletes and pregnant women (physiological hypertrophy). This physiological hypertrophy is reversible and does not decompensate to heart failure, whereas, hypertrophy associated to cardiovascular diseases (pathological hypertrophy) may lead to heart decompensation, eventually resulting in ventricular dilation and HF [8]. Another major feature of cardiac remodeling is the occurrence of cardiac fibrosis, which is the excess deposition of collagens and other extracellular matrix (ECM) proteins. Normal fibrillar collagens form a network between different cell types within the myocardium and are essential for maintaining cardiac geometry during systole and diastole of the heart [9, 10]. Abnormal accumulation of collagen stiffens the heart and affects both systolic and diastolic function [11]. Fibrosis also impairs mechano-electric coupling of cardiomyocytes resulting in increased risks of arrhythmias [12, 13]. Furthermore, excessive accumulation of collagen results in reduced

capillary density and increases oxygen diffusion distance, which in turn affects cardiomyocyte metabolism and performance and ultimately deteriorates heart function [4, 14]. It is important to note that fibrosis happens predominantly in pathological hypertrophy and is nearly absent in physiological hypertrophy [15].

Besides hypertrophy and fibrosis, apoptosis and necrosis of cardiomyocytes may also occur in the remodeled heart as well as infiltration of inflammatory cells, and changes in capillary densities [16]. This review will, however, be focused on the cellular and molecular changes in hypertrophy and fibrosis.

2.2 Cardiomyocyte hypertrophy

2.2.1 Cardiomyocytes and hypertrophic stimuli

The heart is predominantly composed of three cell types, cardiomyocytes (30-40% of all cells), fibroblasts (60-70% of all cells) and endothelial cells (1-2%) [17]. In addition several other cell types are present in low numbers, including smooth muscle cells, macrophages, monocytes and others.

Cardiomyocytes are the major functional cells in the heart and as a result of their large cell size build up 70-80% of the total heart mass [18]. It is the contraction of cardiomyocytes that allows the heart to function as a pump. Therefore, changes in cardiomyocyte numbers or function can have major effects on the pump capacity of the heart. Cardiomyocytes are terminally differentiated cells and lose their ability to proliferate after birth. Therefore, although changes in heart rate can compensate for temporal changes in body demands, sustained changes rely on growth (hypertrophy) of cardiomyocytes, rather than on cell proliferation, or vice versa in a decrease in cell size (atrophy) [19].

Cardiomyocyte hypertrophy induction is a complex process, but is basically driven by two different kinds of stimuli, namely biomechanical stress and neurohumoral factors [20]. Biomechanical stress in the heart refers to any stress leading to hemodynamic changes. *In vitro*, cardiomyocyte stretch can trigger cell hypertrophy directly, without obvious contribution of neurohormones, but *in vivo* most of the hemodynamic overload is accompanied by changes in neurohumoral factors [21]. These factors include angiotensin II (AngII), endothelin-1 (ET-1), catecholamines, and growth factors, like IGF-1. These factors can also directly induce hypertrophy in cultured cardiomyocytes *in vitro* [22-26].

2.2.2 Hypertrophic signaling

Neurohormonal factors, like catecholamines, mediate intracellular responses via hormone

specific 7-transmembrane G-protein-coupled receptors (GPCRs) that are present on the cell membrane of cardiomyocytes [27]. GPCRs are intracellular coupled to trimeric G-protein complexes consisting of an α, β and γ subunit. Upon activation of the receptors the α subunit is liberated from the stable βγ complex and both the α and the βγ complex can subsequently activate downstream effectors (Figure 1) [28]. The ET-1, AngII and α-adrenergic GPCRs are intracellular coupled to the α subunits G_q or G_{11} (Figure 1). Stimulation of the GPCRs liberates $G_{q/11}$ which activates phospholipase C (PLC) resulting in the generation of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2) (Figure 1). DAG subsequently stimulates protein kinase C (PKC), which on its turn activates downstream signaling pathways, like the Mitogen Activated Protein Kinase (MAPK) pathway [29].

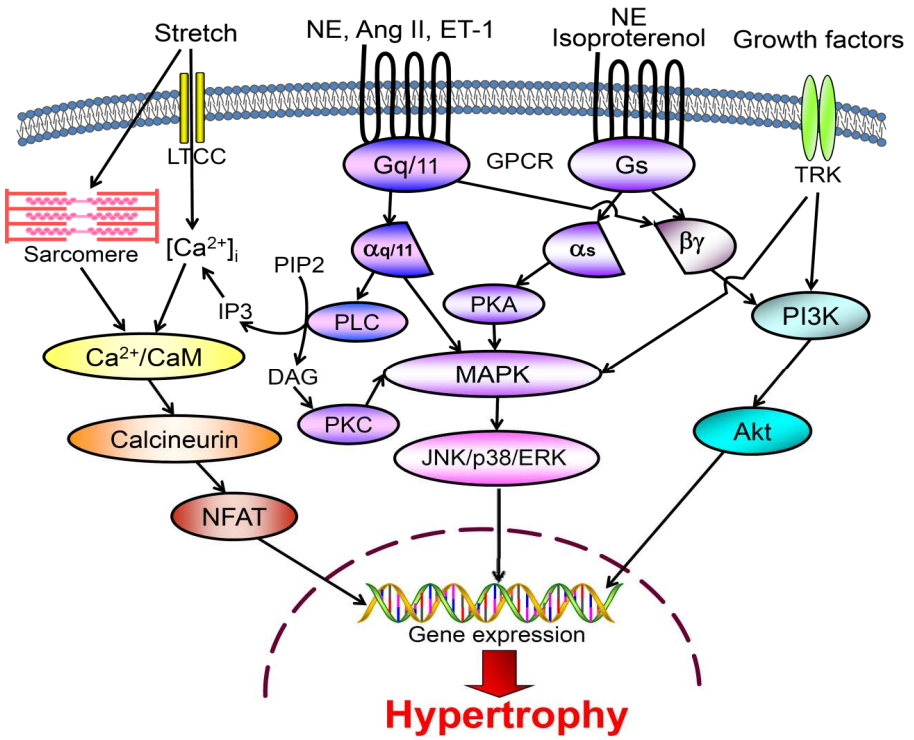


Figure 1. Simplified depiction of signal transduction pathways controlling gene expression and cardiomyocyte hypertrophy. JNK, c-Jun N-terminal kinases; ERK, extracellular signal-regulated kinases; TRK, tyrosine receptor kinase; NE, norepinephrine; LTCC, L-type calcium channel. Other abbreviations are mentioned in the text.

IP3 on the other hand enhances liberation of Ca^{2+} from intracellular stores and hence increases Ca^{2+} / Calmodulin (CaM) levels resulting in the activation of amongst others the Calcineurin / Nuclear Factor of Activated T-cells (NFAT) pathway [30]. In parallel the $G_{\beta\gamma}$ dimer complex can activate PI-3-Kinase (PI3K) resulting in the activation of the Akt (also termed PKB) kinase pathway [31]. The activation of these pathways will increase protein synthesis and activate transcription factors finally resulting in hypertrophy development.

Also β-adrenergic signaling is mediated via GPCRs and the β1-receptor is the most prominent β-adrenergic receptor in the adult heart. In contrast to the afore mentioned GPCRs this receptor is coupled to G_S and activates adenylate cyclase resulting in elevated cyclic-AMP (cAMP) levels and activation of protein kinase A (Figure 1) [32]. PKA can phosphorylate a number of proteins, including the ryanodine receptor (RyR), phospholamban (PLB) and troponin I, resulting in increased contraction and relaxation rates of the heart. However, it also activates directly or indirectly pathways that induce cardiomyocyte hypertrophy (Figure 1). In addition to the β1-receptor the heart also contains a β2-receptor and this receptor is coupled to both Gs and to Gi. The latter can inhibit adenylate cyclase and hence could counteract the effect of the β1-receptor [33]. In this respect it is also important to note that in HF the ratio of β1 to β2-receptors is shifting in the favor of the latter [34].

Whereas the neurohormonal factors act via GPCRs, most growth factors, like IGF-1 or PDGF, act via tyrosine protein kinase receptors [35]. Binding of the particular growth hormone to its receptor result in the activation of these tyrosine kinase receptors and binding of numerous scaffolds to these receptors. This finally leads to the activation of number of downstream signaling pathways, including the MAPK and PI-3-K/Akt pathways and hence in hypertrophy development (Figure 1). Although neurohormonal and growth factor signal transduction pathways have been explored in great detail, biomechanical sensing pathways are still less understood. Biomechanical stretch sensing includes stretch-activated ion channels, cell surface receptors such as integrins, cadherin complexes involved in cell-cell adhesions and signaling from the sarcomere [36, 37]. Again, this result in the activation of a wide range of signaling pathways, including the above mentioned hypertrophy inducing pathways (Figure 1).

Although all signaling pathways are often depicted as linear pathways there is a vast amount of cross-talk between these pathways. Moreover, there is a precise temporal and spatial control of these pathways and the presence of numerous subtypes of the different signaling proteins (e.g. PLCβ, γ, δ, ε, ζ, η) generates and much more complex and dynamic situation as depicted in Figure 1. It is the intricate balance between these different pathways that ultimately will determine the development of cardiomyocyte hypertrophy.

2.2.3 Modulating major hypertrophic signalling pathways in animal models

Despite the detailed investigations of the molecular pathways in cardiomyocyte hypertrophy, so far no treatments options have been generated focused on these pathways. One reason for this is the fact that these pathways do not appear to be simple on/off switches, but instead a delicate balance in activities appears to determine the downstream effects. For example, Shiojima et al.[38] reported that transgenic mice overexpressing Akt-1 showed modest growth of the heart in the short term similar to physiological hypertrophy. However, sustained long term overexpression generated excessive cardiac hypertrophy contributing to heart failure in these mice due to inhibition of coronary angiogenesis [38]. Akt1-deficient mice on the other hand did not show any specific cardiac phenotype under normal conditions [39]. Surprisingly, however, DeBosch et al found that in response to a TAC-operation Akt1-deficient mice had higher ANP and β-MHC expression and a higher heart weight due to cardiomyocyte hypertrophy, as compared to wild type mice [39]. Thus depending on the condition, both low and high Akt1 activity could induce cardiac hypertrophy. Interestingly, exercise mediated hypertrophy was absent in Akt1 deficient mice indicating that Akt1 is essential for physiological, but not pathological hypertrophy. Together, these results show that the strength and duration of Akt1 signaling appears to determine whether this results in physiological or pathological hypertrophy, or can even suppress growth in certain conditions. This exemplifies the complexity and sensitivity of the system and shows that one should be careful with the interpretation of deletion and overexpression studies.

Compared to PI3K/Akt signaling, the calcineurin/NFAT signaling pathway seems to be specifically linked to pathological cardiac hypertrophy. Calcineurin activity and protein levels are upregulated in heart tissue from both exercise- and TAC-induced cardiac hypertrophy models, as well as in dilated heart failure [40, 41]. Transgenic mice expressing a truncated constitutively active form of calcineurin demonstrated a 2-3 fold increase in heart size that rapidly progressed to dilated heart failure within 3 months [42]. Mice with deficient calcineurin-β showed resistance to diverse hypertrophic stimuli, suggesting that calcineurin functions as a central regulator of cardiac hypertrophy resulting from these stressors [43]. However, recent results show that overexpression of a calcineurin splice variant (β1) improves cardiac function and attenuates hypertrophy post-MI [44]. This again exemplifies the complexity of these pathways. Using the calcineurin inhibitor cyclosporine pressure-induced cardiac hypertrophy could be suppressed in mice [45, 46]. However, despite attenuation of pressure overload induced hypertrophy, it has also been shown to enhance susceptibility to decompensation and HF [47]. Moreover, since cyclopsporine is a strong immunosuppressant, by targeting NFAT in T cells its use in a clinical setting for treating HF will not to be feasible.

Thus, although this pathway could be a potential target, its prominent role in many other cell types will make it difficult to be used as a HF therapy.

Also the mitogen-activated protein kinases (MAPK) signaling pathways, in particular the ERK pathway has been linked to cardiac hypertrophy and ERK activity is induced during HF. Transgenic mice with increased activity of ERK 1/2 showed significant cardiac hypertrophy [48] and activation of ERK1/2 induced more cardiac hypertrophy following TAC [49]. However, because of its importance in many different cellular processes and its intertwining with many other pathways it will be difficult to generate specific HF therapeutic approaches around this pathway. Therefore, pharmaceutical targeting of these signaling pathways to treat HF might be more difficult than originally anticipated. Downstream targets of these main pathways, which could potentially allow more specific and more subtle targeting, may therefore comprise altenative targets.

2.2.4 Gene expression changes in cardiac hypertrophy and the fetal gene program

The intracellular signaling pathways that are triggered by hypertrophic stimuli transduce these signals to the nucleus resulting in altered gene expression patterns (Figure 1). The downstream transcription factors of these pathways are critical to regulate hypertrophy specific gene expression. These transcription factors include GATA4 [50], GATA6 [51], NFAT [52], Nkx2.5 [53], nuclear factor κB (NFκB) [54], myocyte enhancer factor 2 (MEF2) [55], serum response factor (SRF) [56], Brg1 [57], Hand 1/2 [58] and Smad transcription factors [59]. Many of these transcription factors are thought to mediate the reactivation of the "fetal gene program" [60, 61]. The fetal gene program refers to the fetal gene profile that returns in the adult cardiomyocyte in response to stress triggers like pressure overload, myocardial infarction, cardiac arrhythmias, valvular disease, and genetic mutations in contractile proteins [62]. One characteristic of the fetal gene program is the re-expression of atrial natriuretic peptide (ANP (NPPA)) amongst others (see Table 1). During development the expression of ANP is an early hallmark for the differentiating working myocardium of the atria and ventricles of the heart. ANP expression is down-regulated in ventricles at the end of fetal development and around the time of birth ventricular ANP expression is further downregulated to very low levels, while atrial expression remains high [63, 64]. During HF ANP expression is reinduced in the adult ventricle. A large number of regulatory elements were found in the 700 bp proximal promoter fragment, including binding sites for GATA4-6, Mef2c, SRF, Nkx2.5 and others [65]. *In vivo* analysis revealed, however, that fetal ventricular activity is provided by region between -127 to -27 kb, whereas adult re-expression in the ventricle requires regulatory sequences located between -27 to -11 and/or +5 to +58 kb [66]. Thus, fetal expression and

adult re-expression of ANP during HF are mediated by different promoter elements. This results show that divergent regulatory pathways may activate fetal gene expression in the fetal heart versus the failing adult heart. The term “fetal gene program” might therefore be somewhat misleading. These results also show that proximal promoter binding studies should be taken with caution and that the *in vivo* situation might be much more complex.

Table 1. A selection of genes belonging to the fetal gene program

Genes	Adult	Fetal	Hypertrophy
α-MHC	↑	↓	↓
β-MHC	↓	↑	↑
SERCA2	↑	↓	↓
ANP	↓	↑	↑
BNP	↓	↑	↑
α-SKA	↓	↑	↑
GLUT4	↑	↓	↓

Arrows indicate increased or decreased expression.

2.2.5 Beneficial and detrimental gene expression changes

As mentioned above ANP expression is reactivated in the failing myocardium. ANP is a circulating hormone excreted by the heart and plays an important role in the regulation of fluid volume and blood pressure [67, 68]. ANP, as well as BNP, functions to reduce the extra fluid from the circulatory system by inhibition of renal tubular NaCl reabsorbtion [69]. The absence of ANP results in salt-sensitive hypertension and cardiac hypertrophy in ANP knock-out mice [70]. Whereas ANP transgenic mice exihibit lower arterial blood pressure; in acute blood volume expansion generated by intravenous infusion, the mice show enhanced diuretic and natriuretic responses [71,72]. ANP has therefore a positive effect since it reduces blood pressure and unloads the failing heart.

During cardiac hypertrophy and HF the main myocardial energy substrate switches from fatty acids to carbohydrates. This switch reflects the fetal situation in which glucose is the main metabolic substrate of the heart. Also at the gene expression level changes in numerous metabolic enzymes has been observed, although it appears that both glucose and fatty acid metabolism encoding genes are downregulated [73]. Since the changes in gene expression of some metabolic enzymes do not directly correlate with changes in enzyme levels and activities it is still under debate whether these changes directly contribute to this switch [74]. Since carbohydrate metabolism requires less oxygen as compared to fatty acid metabolism, this switch is often considered to be beneficial, at least for the ischemic heart. Emerging evidence shows that this fetal pattern metabolic shift can improve the contractile

function of the failing heart [75]. Inhibition of fatty acid β-oxidation by Trimetazidine (1-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride) has been reported to improve left ventricular function in patients with chronic HF [76, 77]. The beneficial impact shown by these small clinical trials suggests that targeting metabolism could be a promising strategy for HF and hence deserves further investigations.

Cardiac remodeling is also accompanied by changes in gene expression of contractile sarcomere proteins, in particular the myosin heavy chains (MHC). Two types of MHC, α- and β, are specifically expressed in the heart [78, 79]. α-MHC has a higher adenosine triphosphatase activity and higher filament sliding velocity than β-MHC, but the latter can generate cross-bridge force with a higher economy of energy [80, 81]. The heart contractile performance is correlated with the relative amount of each MHC. More α-MHC expression results in a increased contractile velocity, whereas hearts with a higher β-MHC content generate force in a more economic way [82]. In rodent ventricles more than 90% of MHC is α-MHC [83-85]. Several investigators have reported an increase in β-MHC and decrease in the α-MHC isoform in hypertrophied ventricles [86, 87] and in heart failure. The level of β-MHC closely correlates with the degree of cardiac hypertrophy [85]. Although the shift from α- to β-MHC can preserve energy, increased β-MHC also reduces the velocity of shortening, which may have a detrimental effect on failing hearts and promote disease progression [88, 89]. Although in human β-MHC is the predominant form in the heart, as opposed to rodents, also in humans β-MHC expression increases during HF, although these effects are less pronounced as in rodents [90, 91].

Although the examples above indicate predominantly beneficial effects for the fetal gene program, other changes appear to be detrimental. One example is the downregulation of the Ca^{2+} activated sarcoplasmic/endo-plasmic reticulum ATPase (SERCA). SERCA is the main transporter for Ca^{2+} from the cytoplasm to the sarcoplasmic/endoplasmic reticulum [92]. Using ATP as an energy source, SERCA transports Ca^{2+} form the plasma to the sarcoplasmic reticulum in order to maintain calcium homeostasis within cells. This process is essential for calcium handling and excitation-contraction coupling in cardiac and skeletal muscles. In mammals, three isoforms of SERCA have been identified, among them SERCA2 is most prominently expressed in the heart. SERCA2 mRNA increases markedly after birth, sustains at high levels for a long period in young rodents and decreases in ageing animals thereafter [93]. Whereas during initial stages of hypertrophy development SERCA2 expression (and activity) increases and may improve the function of the hypertrophied rat hearts [94], in heart failure, SERCA2 decreases at mRNA and protein level [95-99]. It has been reported that the decrease of SERCA2 (activity) contributes to the functional abnormalities in the ageing heart. Schmidt U and coworkers increased the SERCA2a level in cardiomyocytes by

adenovirus-mediated gene transfer and observed that increased levels of SERCA2a can improve heart function in ageing rat [100]. Moreover, the calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID) phase 1/2 study that enrolled a total of 39 patients with severe heart failure randomized to adeno-associated virus-mediated transfer of SERCA2a or placebo has recently been presented [101].The CUPID trial suggests adequate safety and potential efficacy warranting further studies to evaluate this new treatment option in heart failure [102]. This also indicates that gene or pharmaceutical targeting of fetal gene program components could potentially attenuate hypertrophy and hence might provide new therapeutic targets.

2.3 Fibrosis

2.3.1 The extracellular matrix and fibrosis

Cardiac fibrosis is a hallmark of pathological hypertrophy and contributes to heart failure progression. Fibrosis is characterized by disproportional accumulation of extracellular matrix (ECM) componts The ECM is composed of elastin, fibrillin, fibronectin, proteoglycans, basement membranes and fibrillar collagen [103]. The synthesis and degradation of ECM in the heart is a dynamic process. It is estimated that collagens in the heart are renewed at a rate of 0.6% per day with an average half-life of 80-120 days [104]. The major function of the ECM is to provide a structural backbone to preserve tissue architecture in both systolic and diastolic phase. During systole, the ECM transmits force generated by single cardiomyocytes into organized cardiac contraction. Whereas during diastole, the ECM network prevents cardiomyocyte overstretch and slippage, and hence protects the heart against deformation [105]. Due to its structural functions, ECM was initially considered less important except for reparative (replacement) fibrosis in which matrix components appear at sites of cell loss. However, besides the structural functions, the ECM also serves as modulator of growth, tissue differentiation, and angiogenesis [106, 107]. Studies from animals and patients indicate there is another type of fibrosis, reactive fibrosis, which occurs in the cardiac interstitium and peri-vascular area in the absence of myocyte loss [104]. This reactive fibrosis is an adaptive response to preserve the force generating capacity of the hypertrophied heart, especially in the beginning of disease development. However, excessive fibrosis can disturb normal contractility and cardiomyocyte function. The amount of fibrosis is dependent on changes in the balance of matrix formation and degradation. In addition, the ratio between the different matrix components and the degree of collagen crosslinking are associated with cardiac function. This is not only because of changes in stiffness of the matrix, but rearrangements of the collagen network also affect the conductance of electrical signals [108, 109].

2.3.2 Fibroblasts and myofibroblasts in fibrosis

Fibroblasts play an essential role in generating the extracellular matrix and constitute about 60-70% of all cells in the heart, but due to their relative small size these cells only make up 20-30% of the heart mass. Fibroblasts are poorly defined cells (no specific markers) and are present in all tissues in the body. These cells do, however, contain certain proteins absent in cardiomyocytes, including vimentin and the collagen receptor Discoid domain receptor 2 [108]. Fibroblasts, in particular myofibroblasts, secrete large amounts of extracellular matrix components, like collagens, fibronectin, eleastins, as well as enzymes modulating the ECM, like matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs)[110].

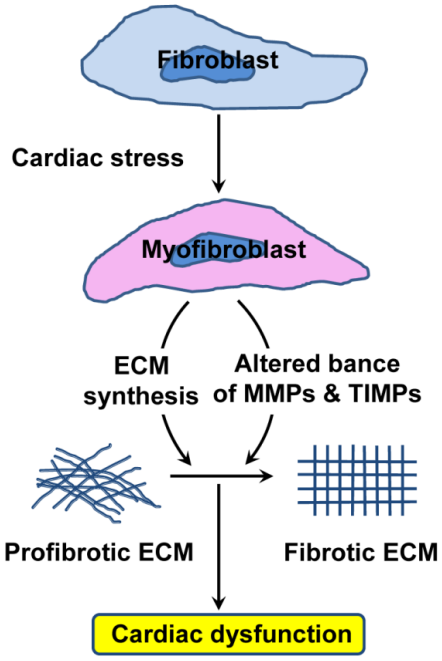


Figure 2 Fibroblast to myofibroblast transition and ECM modulation. Fibroblasts transform to myofibroblasts in response to cardiac stress signals, including mechanical stretch, hormones and cytokines. The myofibroblasts have increased capacity of ECM synthesis and can also alter the balance of MMPs and TIMPs to promote fibrosis. These alterations contribute to cardiac dysfunction. Figure adapted from Berk BC, et al. *J Clin Invest*. 2007.[103]

Under pathological conditions fibroblasts tansform into an active phenotype called myofibroblasts, which is essential for the development of cardiac fibrosis (Figure 2) [111]. This myofibroblasts presents characteristics in between those of fibroblasts and smooth muscle cells [112]. In culture this differentiation can be induced by stretch and inflammatory mediators present in the diseased heart, like TGF-β and IL-1 and IL-6 and differentiation requires adhesion and integrin signaling [113].The steps in this process are not well understood and, in particular, the molecular mechanisms by which this differentiation occurs *in vivo* are unknown.

As compared to fibroblasts, myofibroblasts show increased migration activity and increased collagen synthesis and proinflammatory cytokine secretion as compared to fibroblasts. These cells express several smooth muscle markers (early differentiation markers), such as smooth muscle α-actin, tropomyosin, nonmuscle myosin heavy chain-B (SMemb), and smooth muscle 22α [114-116]. Besides being a marker for the fibroblast to myofibroblast switch, α-SMA has been indicated to have contractile properties and play an important role in the generation of myofibroblast contractile force [117]. More stringent smooth muscle markers (late differentiation markers) like smooth muscle myosin heavy chains are not expressed in myofibroblasts [118].

2.3.3 Changes in (myo)fibroblast gene expression during HF

In the healthy heart expression and secretion of matrix components is mainly dependent on fibroblasts in the heart, whereas in the stressed heart the myofibroblast becomes the major source for the production of matrix components. The hypersecretory ability of this cell type requires altered expression of a number of matrix genes including collagen type I and III (Col I and III). Col I and Col III represent about 80% and 10% of the total ECM in the heart, respectively [104]. Col I gives the heart its tensile strength and makes the myocardium stiff when the level is increased. Col III has a resilience ability that keeps the structural integrity and distensibility of the heart [104]. Changes in collagen expression occur during HF development and the ratio of these differences might provide information about the type of dysfunction [119]. Kasner et al. for example observed that both mRNA and protein expression of Col I, but not Col III, were increased in diastolic HF patients with normal systolic function and the Col I level correlated with the diastolic dysfunction [120].

Similar to the different collagen isoforms, also a delicate balance exists between multiple metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Since the turnover of ECM in heart tissue depends principally on these proteins, changes in relative expressions and activities will affect matrix formation. MMPs are secreted as inactive precursors (Pro-MMP) which must be activated by proteolytic cleavage of the amino terminal propeptide domain. *In vitro*, inflammatory hormones, like IL-1 appear to downregulate expression of MMPs, whereas profibrotic hormones like angiotensin II decrease expression of several MMPs. *In vivo*, after MI, MMP1 expression is initially increased and collagen degradation exceeds synthesis [121]. At later stages, however, ECM synthesis exceeds degradation and MMP expression is decreased, whereas TIMP expression is increased, resulting in a decreased MMP/TIMP ratio [121]. Interestingly, TIMP-1 knockout mice demonstrated less myocardial fibrillar collagen content in association with increased left ventricle end-diastolic volume and heart mass [122]. Similar effects were indicated in TIMP-3

deficient mice [123]. In addition to the direct inhibition of MMPs, the TIMPs also have influence on fibroblast that lead to collagen accumulation. Adenoviral mediated TIMPs overexpression in fibroblast indicated that all TIMPs can promote fibroblast proliferation and induce the fibroblast to myofibroblast switch [124].

In summary, pathological stress on the heart results in a switch from fibroblasts to myofibroblasts, which have a phenotype in between fibroblasts and smooth muscle cells and express α-SMA. These cells express and hyper secrete a large variety of matrix components that contribute to matrix remodeling and contribute to HF development. Matrix proteins, like MMPs, TIMPs and collagen breakdown products, could be interesting biomarkers [125] and moreover might constitute interesting targets for HF treatment.

Conclusions

Cardiac stress induces major changes in gene programs, generating cardiomyocyte hypertrophy and fibrosis, which drive cardiac remodeling. Although major signaling pathways have been identified in these processes, targeting these pathways has, so far been unsuccessful in HF treatment. Targeting so called fetal gene program targets might be more rewarding, as exemplified by SERCA2a. Restoring the expression of SERCA2a during HF improves cardiac function and although this requires gene therapy this might be a rewarding approach. Identification and exploration of other fetal gene program genes could therefore be a fruitful enterprise. In addition, controlling fibrotic processes by influencing matrix remodeling genes and enzymes could provide an additional treatment strategy that requires further exploration.

References

1. Zannad F, Briancon S, Juilliere Y, Mertes PM, Villemot JP, Alla F, Virion JM. Incidence, clinical and etiologic features, and outcomes of advanced chronic heart failure: The EPICAL study. epidemiologie de l'insuffisance cardiaque avancee en lorraine. *J Am Coll Cardiol*. 1999;33:734-742.

2. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, Stromberg A, van Veldhuisen DJ, Atar D, Hoes AW, Keren A, Mebazaa A, Nieminen M, Priori SG, Swedberg K, ESC Committee for Practice Guidelines (CPG). ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: The task force for the diagnosis and treatment of acute and chronic heart failure 2008 of the european society of cardiology. developed in collaboration with the heart failure association of the ESC (HFA) and endorsed by the european society of intensive care medicine (ESICM). *Eur J Heart Fail*. 2008;10:933-989.

3. Cohn JN, Ferrari R, Sharpe N. Cardiac remodeling--concepts and clinical implications: A consensus paper from an international forum on cardiac remodeling. behalf of an international forum on cardiac remodeling. *J Am Coll Cardiol*. 2000;35:569-582.

4. Sabbah HN, Goldstein S. Ventricular remodelling: Consequences and therapy. *Eur Heart J*. 1993;14 Suppl C:24-29.

5. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. experimental observations and clinical implications. *Circulation*. 1990;81:1161-1172.

6. Ruwhof C, van der Laarse A. Mechanical stress-induced cardiac hypertrophy: Mechanisms and signal transduction pathways. *Cardiovasc Res*. 2000;47:23-37.

7. Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR, Doetschman T. TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. *J Clin Invest*. 2002;109:787-796.

8. Drazner MH. The progression of hypertensive heart disease. *Circulation*. 2011;123:327-334.

9. Woodiwiss AJ, Tsotetsi OJ, Sprott S, Lancaster EJ, Mela T, Chung ES, Meyer TE, Norton GR. Reduction in myocardial collagen cross-linking parallels left ventricular dilatation in rat models of systolic chamber dysfunction. *Circulation*. 2001;103:155-160.

10. Kim HE, Dalal SS, Young E, Legato MJ, Weisfeldt ML, D'Armiento J. Disruption of the myocardial extracellular matrix leads to cardiac dysfunction. *J Clin Invest*. 2000;106:857-866.

11. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. fibrosis and renin-angiotensin-aldosterone system. *Circulation*. 1991;83:1849-1865.

12. de Bakker JM, van Capelle FJ, Janse MJ, Tasseron S, Vermeulen JT, de Jonge N, Lahpor JR. Fractionated electrograms in dilated cardiomyopathy: Origin and relation to abnormal conduction. *J Am Coll Cardiol*. 1996;27:1071-1078.

13. Thompson SA, Copeland CR, Reich DH, Tung L. Mechanical coupling between myofibroblasts and cardiomyocytes slows electric conduction in fibrotic cell monolayers. *Circulation*. 2011;123:2083-2093.

14. Schnee JM, Hsueh WA. Angiotensin II, adhesion, and cardiac fibrosis. *Cardiovasc Res*. 2000;46:264-268.

15. Hill JA, Olson EN. Cardiac plasticity. *N Engl J Med*. 2008;358:1370-1380.

16. De Boer RA, Pinto YM, Van Veldhuisen DJ. The imbalance between oxygen demand and supply as a potential mechanism in the pathophysiology of heart failure: The role of microvascular growth and abnormalities. *Microcirculation*. 2003;10:113-126.

17. Nag AC. Study of non-muscle cells of the adult mammalian heart: A fine structural analysis and distribution. *Cytobios*. 1980;28:41-61.

18. McMullen JR, Jennings GL. Differences between pathological and physiological cardiac hypertrophy: Novel therapeutic strategies to treat heart failure. *Clin Exp Pharmacol Physiol*. 2007;34:255-262.

19. Tam SK, Gu W, Mahdavi V, Nadal-Ginard B. Cardiac myocyte terminal differentiation. potential for cardiac regeneration. *Ann N Y Acad Sci*. 1995;752:72-79.

20. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol*. 2006;7:589-600.

21. Yamazaki T, Komuro I, Yazaki Y. Molecular mechanism of cardiac cellular hypertrophy by mechanical stress. *J Mol Cell Cardiol*. 1995;27:133-140.

22. Sadoshima J, Izumo S. Signal transduction pathways of angiotensin II--induced c-fos gene expression in cardiac myocytes in vitro. roles of phospholipid-derived second messengers. *Circ Res*. 1993;73:424-438.

23. Asakawa M, Takano H, Nagai T, Uozumi H, Hasegawa H, Kubota N, Saito T, Masuda Y, Kadowaki T, Komuro I. Peroxisome proliferator-activated receptor gamma plays a critical role in inhibition of cardiac hypertrophy in vitro and in vivo. *Circulation*. 2002;105:1240-1246.

24. Ito H, Hiroe M, Hirata Y, Tsujino M, Adachi S, Shichiri M, Koike A, Nogami A, Marumo F. Insulin-like growth factor-I induces hypertrophy with enhanced expression of muscle specific genes in cultured rat cardiomyocytes. *Circulation*. 1993;87:1715-1721.

25. Irukayama-Tomobe Y, Miyauchi T, Sakai S, Kasuya Y, Ogata T, Takanashi M, Iemitsu M, Sudo T, Goto K, Yamaguchi I. Endothelin-1-induced cardiac hypertrophy is inhibited by activation of peroxisome proliferator-activated receptor-alpha partly via blockade of c-jun NH2-terminal kinase pathway. *Circulation*. 2004;109:904-910.

26. Liao Y, Asakura M, Takashima S, Ogai A, Asano Y, Shintani Y, Minamino T, Asanuma H, Sanada S, Kim J, Kitamura S, Tomoike H, Hori M, Kitakaze M. Celiprolol, a vasodilatory beta-blocker, inhibits pressure overload-induced cardiac hypertrophy and prevents the transition to heart failure via nitric oxide-dependent mechanisms in mice. *Circulation*. 2004;110:692-699.

27. Clerk A, Sugden PH. Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists. *Am J Cardiol*. 1999;83:64H-69H.

28. Wettschureck N, Offermanns S. Mammalian G proteins and their cell type specific functions. *Physiol Rev*. 2005;85:1159-1204.

29. Akhter SA, Luttrell LM, Rockman HA, Iaccarino G, Lefkowitz RJ, Koch WJ. Targeting the receptor-gq interface to inhibit in vivo pressure overload myocardial hypertrophy. *Science*. 1998;280:574-577.

30. Molkentin JD. Calcineurin-NFAT signaling regulates the cardiac hypertrophic response in coordination with the MAPKs. *Cardiovasc Res*. 2004;63:467-475.

31. Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol*. 2004;37:449-471.

32. Xiao RP. Beta-adrenergic signaling in the heart: Dual coupling of the beta2-adrenergic receptor to G(s) and G(i) proteins. *Sci STKE*. 2001;2001:re15.

33. Xiao RP, Ji X, Lakatta EG. Functional coupling of the beta 2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol Pharmacol*. 1995;47:322-329.

34. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res*. 2003;93:896-906.

35. Hausenloy DJ, Yellon DM. Cardioprotective growth factors. *Cardiovasc Res*. 2009;83:179-194.

36. Lammerding J, Kamm RD, Lee RT. Mechanotransduction in cardiac myocytes. *Ann N Y Acad Sci*. 2004;1015:53-70.

37. Gao M, Sotomayor M, Villa E, Lee EH, Schulten K. Molecular mechanisms of cellular mechanics. *Phys Chem Chem Phys*. 2006;8:3692-3706.

38. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest*. 2005;115:2108-2118.

39. DeBosch B, Treskov I, Lupu TS, Weinheimer C, Kovacs A, Courtois M, Muslin AJ. Akt1 is required for physiological cardiac growth. *Circulation*. 2006;113:2097-2104.

40. Lim HW, De Windt LJ, Steinberg L, Taigen T, Witt SA, Kimball TR, Molkentin JD. Calcineurin expression, activation, and function in cardiac pressure-overload hypertrophy. *Circulation*. 2000;101:2431-2437.

41. Eto Y, Yonekura K, Sonoda M, Arai N, Sata M, Sugiura S, Takenaka K, Gualberto A, Hixon ML, Wagner MW, Aoyagi T. Calcineurin is activated in rat hearts with physiological left ventricular hypertrophy induced by voluntary exercise training. *Circulation*. 2000;101:2134-2137.

42. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. 1998;93:215-228.

43. Bueno OF, Wilkins BJ, Tymitz KM, Glascock BJ, Kimball TF, Lorenz JN, Molkentin JD. Impaired cardiac hypertrophic response in calcineurin abeta -deficient mice. *Proc Natl Acad Sci U S A*. 2002;99:4586-4591.

44. Felkin LE, Narita T, Germack R, Shintani Y, Takahashi K, Sarathchandra P, Lopez-Olaneta MM, Gomez-Salinero JM, Suzuki K, Barton PJ, Rosenthal N, Lara-Pezzi E. Calcineurin splicing variant calcineurin Abeta1 improves cardiac function after myocardial infarction without inducing hypertrophy. *Circulation*. 2011;123:2838-2847.

45. Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, Colbert MC, Gualberto A, Wieczorek DF, Molkentin JD. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. *Science*. 1998;281:1690-1693.

46. Semeniuk LM, Severson DL, Kryski AJ, Swirp SL, Molkentin JD, Duff HJ. Time-dependent systolic and diastolic function in mice overexpressing calcineurin. *Am J Physiol Heart Circ Physiol*. 2003;284:H425-30.

47. Meguro T, Hong C, Asai K, Takagi G, McKinsey TA, Olson EN, Vatner SF. Cyclosporine attenuates pressure-overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. *Circ Res*. 1999;84:735-740.

48. Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN, Molkentin JD. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. *EMBO J*. 2000;19:6341-6350.

49. Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, Braunwart J, Glascock BJ, Klevitsky R, Kimball TF, Hewett TE, Molkentin JD. Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. *J Clin Invest*. 2003;111:1475-1486.

50. Hasegawa K, Lee SJ, Jobe SM, Markham BE, Kitsis RN. Cis-acting sequences that mediate induction of beta-myosin heavy chain gene expression during left ventricular hypertrophy due to aortic constriction. *Circulation*. 1997;96:3943-3953.

51. van Berlo JH, Elrod JW, van den Hoogenhof MM, York AJ, Aronow BJ, Duncan SA, Molkentin JD. The transcription factor GATA-6 regulates pathological cardiac hypertrophy. *Circ Res*. 2010;107:1032-1040.

52. Vega RB, Bassel-Duby R, Olson EN. Control of cardiac growth and function by calcineurin signaling. *J Biol Chem*. 2003;278:36981-36984.

53. Warren SA, Terada R, Briggs LE, Cole-Jeffrey CT, Chien WM, Seki T, Weinberg EO, Yang TP, Chin MT, Bungert J, Kasahara H. Differential role of Nkx2-5 in activation of the atrial natriuretic factor gene in the developing versus failing heart. *Mol Cell Biol*. 2011;31:4633-4645.

54. Freund C, Schmidt-Ullrich R, Baurand A, Dunger S, Schneider W, Loser P, El-Jamali A, Dietz R, Scheidereit C, Bergmann MW. Requirement of nuclear factor-kappaB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. *Circulation*. 2005;111:2319-2325.

55. Kolodziejczyk SM, Wang L, Balazsi K, DeRepentigny Y, Kothary R, Megeney LA. MEF2 is upregulated during cardiac hypertrophy and is required for normal post-natal growth of the myocardium. *Curr Biol*. 1999;9:1203-1206.

56. Paradis P, MacLellan WR, Belaguli NS, Schwartz RJ, Schneider MD. Serum response factor mediates AP-1-dependent induction of the skeletal alpha-actin promoter in ventricular myocytes. *J Biol Chem*. 1996;271:10827-10833.

57. Hang CT, Yang J, Han P, Cheng HL, Shang C, Ashley E, Zhou B, Chang CP. Chromatin regulation by Brg1 underlies heart muscle development and disease. *Nature*. 2010;466:62-67.

58. Risebro CA, Smart N, Dupays L, Breckenridge R, Mohun TJ, Riley PR. Hand1 regulates cardiomyocyte proliferation versus differentiation in the developing heart. *Development*. 2006;133:4595-4606.

59. Akazawa H, Komuro I. Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res*. 2003;92:1079-1088.

60. Oka T, Xu J, Molkentin JD. Re-employment of developmental transcription factors in adult heart disease. *Semin Cell Dev Biol*. 2007;18:117-131.

61. Frey N, Olson EN. Cardiac hypertrophy: The good, the bad, and the ugly. *Annu Rev Physiol*. 2003;65:45-79.

62. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: A strong hypothesis. *Heart Fail Rev*. 2007;12:331-343.

63. Bloch KD, Seidman JG, Naftilan JD, Fallon JT, Seidman CE. Neonatal atria and ventricles secrete atrial natriuretic factor via tissue-specific secretory pathways. *Cell*. 1986;47:695-702.

64. Seidman CE, Schmidt EV, Seidman JG. Cis-dominance of rat atrial natriuretic factor gene regulatory sequences in transgenic mice. *Can J Physiol Pharmacol*. 1991;69:1486-1492.

65. Houweling AC, van Borren MM, Moorman AF, Christoffels VM. Expression and regulation of the atrial natriuretic factor encoding gene nppa during development and disease. *Cardiovasc Res*. 2005;67:583-593.

66. Horsthuis T, Houweling AC, Habets PE, de Lange FJ, el Azzouzi H, Clout DE, Moorman AF, Christoffels VM. Distinct regulation of developmental and heart disease-induced atrial natriuretic factor expression by two separate distal sequences. *Circ Res*. 2008;102:849-859.

67. Ballermann BJ, Brenner BM. George E. brown memorial lecture. role of atrial peptides in body fluid homeostasis. *Circ Res*. 1986;58:619-630.

68. Yasue H, Obata K, Okumura K, Kurose M, Ogawa H, Matsuyama K, Jougasaki M, Saito Y, Nakao K, Imura H. Increased secretion of atrial natriuretic polypeptide from the left ventricle in patients with dilated cardiomyopathy. *J Clin Invest*. 1989;83:46-51.

69. Dietz JR. Mechanisms of atrial natriuretic peptide secretion from the atrium. *Cardiovasc Res*. 2005;68:8-17.

70. John SW, Krege JH, Oliver PM, Hagaman JR, Hodgin JB, Pang SC, Flynn TG, Smithies O. Genetic decreases in atrial natriuretic peptide and salt-sensitive hypertension. *Science*. 1995;267:679-681.

71. Steinhelper ME, Cochrane KL, Field LJ. Hypotension in transgenic mice expressing atrial natriuretic factor fusion genes. *Hypertension*. 1990;16:301-307.

72. Field LJ, Veress AT, Steinhelper ME, Cochrane K, Sonnenberg H. Kidney function in ANF-transgenic mice: Effect of blood volume expansion. *Am J Physiol*. 1991;260:R1-5.

73. Razeghi P, Young ME, Alcorn JL, Moravec CS, Frazier OH, Taegtmeyer H. Metabolic gene expression in fetal and failing human heart. *Circulation*. 2001;104:2923-2931.

74. Morgan EE, Chandler MP, Young ME, McElfresh TA, Kung TA, Rennison JH, Tserng KY, Hoit BD, Stanley WC. Dissociation between gene and protein expression of metabolic enzymes in a rodent model of heart failure. *Eur J Heart Fail*. 2006;8:687-693.

75. Barsotti A, Giannoni A, Di Napoli P, Emdin M. Energy metabolism in the normal and in the diabetic heart. *Curr Pharm Des*. 2009;15:836-840.

76. Vitale C, Wajngaten M, Sposato B, Gebara O, Rossini P, Fini M, Volterrani M, Rosano GM. Trimetazidine improves left ventricular function and quality of life in elderly patients with coronary artery disease. *Eur Heart J*. 2004;25:1814-1821.

77. Fragasso G, Salerno A, Lattuada G, Cuko A, Calori G, Scollo A, Ragogna F, Arioli F, Bassanelli G, Spoladore R, Luzi L, Margonato A, Perseghin G. Effect of partial inhibition of fatty acid oxidation by trimetazidine on whole body energy metabolism in patients with chronic heart failure. *Heart*. 2011;97:1495-1500.

78. Everett AW, Sinha AM, Umeda PK, Jakovcic S, Rabinowitz M, Zak R. Regulation of myosin synthesis by thyroid hormone: Relative change in the alpha- and beta-myosin heavy chain mRNA levels in rabbit heart. *Biochemistry*. 1984;23:1596-1599.

79. Allen DL, Sartorius CA, Sycuro LK, Leinwand LA. Different pathways regulate expression of the skeletal myosin heavy chain genes. *J Biol Chem*. 2001;276:43524-43533.

80. Harris DE, Work SS, Wright RK, Alpert NR, Warshaw DM. Smooth, cardiac and skeletal muscle myosin force and motion generation assessed by cross-bridge mechanical interactions in vitro. *J Muscle Res Cell Motil*. 1994;15:11-19.

81. Sugiura S, Kobayakawa N, Fujita H, Yamashita H, Momomura S, Chaen S, Omata M, Sugi H. Comparison of unitary displacements and forces between 2 cardiac myosin isoforms by the optical trap technique: Molecular basis for cardiac adaptation. *Circ Res*. 1998;82:1029-1034.

82. Alpert NR, Mulieri LA. Increased myothermal economy of isometric force generation in compensated cardiac hypertrophy induced by pulmonary artery constriction in the rabbit. A characterization of heat liberation in normal and hypertrophied right ventricular papillary muscles. *Circ Res*. 1982;50:491-500.

83. Lompre AM, Schwartz K, d'Albis A, Lacombe G, Van Thiem N, Swynghedauw B. Myosin isoenzyme redistribution in chronic heart overload. *Nature*. 1979;282:105-107.

84. Mercadier JJ, Lompre AM, Wisnewsky C, Samuel JL, Bercovici J, Swynghedauw B, Schwartz K. Myosin isoenzyme changes in several models of rat cardiac hypertrophy. *Circ Res*. 1981;49:525-532.

85. Izumo S, Lompre AM, Matsuoka R, Koren G, Schwartz K, Nadal-Ginard B, Mahdavi V. Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. interaction between hemodynamic and thyroid hormone-induced signals. *J Clin Invest*. 1987;79:970-977.

86. Gidh-Jain M, Huang B, Jain P, Gick G, El-Sherif N. Alterations in cardiac gene expression during ventricular remodeling following experimental myocardial infarction. *J Mol Cell Cardiol*. 1998;30:627-637.

87. Ojamaa K, Petrie JF, Balkman C, Hong C, Klein I. Posttranscriptional modification of myosin heavy-chain gene expression in the hypertrophied rat myocardium. *Proc Natl Acad Sci U S A*. 1994;91:3468-3472.

88. Abraham WT, Gilbert EM, Lowes BD, Minobe WA, Larrabee P, Roden RL, Dutcher D, Sederberg J, Lindenfeld JA, Wolfel EE, Shakar SF, Ferguson D, Volkman K, Linseman JV, Quaife RA, Robertson AD, Bristow MR. Coordinate

changes in myosin heavy chain isoform gene expression are selectively associated with alterations in dilated cardiomyopathy phenotype. *Mol Med*. 2002;8:750-760.

89. Fatkin D, McConnell BK, Mudd JO, Semsarian C, Moskowitz IG, Schoen FJ, Giewat M, Seidman CE, Seidman JG. An abnormal ca(2+) response in mutant sarcomere protein-mediated familial hypertrophic cardiomyopathy. *J Clin Invest*. 2000;106:1351-1359.

90. Lowes BD, Gilbert EM, Abraham WT, Minobe WA, Larrabee P, Ferguson D, Wolfel EE, Lindenfeld J, Tsvetkova T, Robertson AD, Quaife RA, Bristow MR. Myocardial gene expression in dilated cardiomyopathy treated with beta-blocking agents. *N Engl J Med*. 2002;346:1357-1365.

91. Lopez JE, Myagmar BE, Swigart PM, Montgomery MD, Haynam S, Bigos M, Rodrigo MC, Simpson PC. Beta-myosin heavy chain is induced by pressure overload in a minor subpopulation of smaller mouse cardiac myocytes. *Circ Res*. 2011;109:629-638.

92. Periasamy M, Bhupathy P, Babu GJ. Regulation of sarcoplasmic reticulum Ca2+ ATPase pump expression and its relevance to cardiac muscle physiology and pathology. *Cardiovasc Res*. 2008;77:265-273.

93. Lompre AM, Lambert F, Lakatta EG, Schwartz K. Expression of sarcoplasmic reticulum ca(2+)-ATPase and calsequestrin genes in rat heart during ontogenic development and aging. *Circ Res*. 1991;69:1380-1388.

94. Miyamoto MI, del Monte F, Schmidt U, DiSalvo TS, Kang ZB, Matsui T, Guerrero JL, Gwathmey JK, Rosenzweig A, Hajjar RJ. Adenoviral gene transfer of SERCA2a improves left-ventricular function in aortic-banded rats in transition to heart failure. *Proc Natl Acad Sci U S A*. 2000;97:793-798.

95. Schwinger RH, Bohm M, Schmidt U, Karczewski P, Bavendiek U, Flesch M, Krause EG, Erdmann E. Unchanged protein levels of SERCA II and phospholamban but reduced Ca2+ uptake and ca(2+)-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. *Circulation*. 1995;92:3220-3228.

96. Studer R, Reinecke H, Bilger J, Eschenhagen T, Bohm M, Hasenfuss G, Just H, Holtz J, Drexler H. Gene expression of the cardiac na(+)-Ca2+ exchanger in end-stage human heart failure. *Circ Res*. 1994;75:443-453.

97. Meyer M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. *Circulation*. 1995;92:778-784.

98. Mercadier JJ, Lompre AM, Duc P, Boheler KR, Fraysse JB, Wisnewsky C, Allen PD, Komajda M, Schwartz K. Altered sarcoplasmic reticulum Ca2(+)-ATPase gene expression in the human ventricle during end-stage heart failure. *J Clin Invest*. 1990;85:305-309.

99. Hasenfuss G, Reinecke H, Studer R, Meyer M, Pieske B, Holtz J, Holubarsch C, Posival H, Just H, Drexler H. Relation between myocardial function and expression of sarcoplasmic reticulum ca(2+)-ATPase in failing and nonfailing human myocardium. *Circ Res*. 1994;75:434-442.

100. Schmidt U, del Monte F, Miyamoto MI, Matsui T, Gwathmey JK, Rosenzweig A, Hajjar RJ. Restoration of diastolic function in senescent rat hearts through adenoviral gene transfer of sarcoplasmic reticulum ca(2+)-ATPase. *Circulation*. 2000;101:790-796.

101. Jaski BE, Jessup ML, Mancini DM, Cappola TP, Pauly DF, Greenberg B, Borow K, Dittrich H, Zsebo KM, Hajjar RJ, Calcium Up-Regulation by Percutaneous Administration of Gene Therapy In Cardiac Disease (CUPID) Trial Investigators. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID trial), a first-in-human phase 1/2 clinical trial. *J Card Fail*. 2009;15:171-181.

102. Hasenfuss G. Gene transfer of the sarcoplasmic reticulum pump in the treatment of heart failure. *Eur J Heart Fail*. 2011;13:245-246.

103. Berk BC, Fujiwara K, Lehoux S. ECM remodeling in hypertensive heart disease. *J Clin Invest*. 2007;117:568-575.

104. Weber KT. Cardiac interstitium in health and disease: The fibrillar collagen network. *J Am Coll Cardiol*. 1989;13:1637-1652.

105. Weber KT, Sun Y, Tyagi SC, Cleutjens JP. Collagen network of the myocardium: Function, structural remodeling and regulatory mechanisms. *J Mol Cell Cardiol*. 1994;26:279-292.

106. Kakkar R, Lee RT. Intramyocardial fibroblast myocyte communication. *Circ Res*. 2010;106:47-57.

107. Silvan U, Diez-Torre A, Arluzea J, Andrade R, Silio M, Arechaga J. Hypoxia and pluripotency in embryonic and embryonal carcinoma stem cell biology. *Differentiation*. 2009;78:159-168.

108. Camelliti P, Borg TK, Kohl P. Structural and functional characterisation of cardiac fibroblasts. *Cardiovasc Res*. 2005;65:40-51.

109. De Jong AM, Maass AH, Oberdorf-Maass SU, Van Veldhuisen DJ, Van Gilst WH, Van Gelder IC. Mechanisms of atrial structural changes caused by stretch occurring before and during early atrial fibrillation. *Cardiovasc Res*. 2011;89:754-765.

110. Porter KE, Turner NA. Cardiac fibroblasts: At the heart of myocardial remodeling. *Pharmacol Ther*. 2009;123:255-278.

111. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol*. 1993;122:103-111.

112. Sappino AP, Schurch W, Gabbiani G. Differentiation repertoire of fibroblastic cells: Expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab Invest*. 1990;63:144-161.

113. Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM, Thomas PE. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem*. 2003;278:12384-12389.

114. Squires CE, Escobar GP, Payne JF, Leonardi RA, Goshorn DK, Sheats NJ, Mains IM, Mingoia JT, Flack EC, Lindsey ML. Altered fibroblast function following myocardial infarction. *J Mol Cell Cardiol*. 2005;39:699-707.

115. Santiago JJ, Dangerfield AL, Rattan SG, Bathe KL, Cunnington RH, Raizman JE, Bedosky KM, Freed DH, Kardami E, Dixon IM. Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: Expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. *Dev Dyn*. 2010;239:1573-1584.

116. Flack EC, Lindsey ML, Squires CE, Kaplan BS, Stroud RE, Clark LL, Escobar PG, Yarbrough WM, Spinale FG. Alterations in cultured myocardial fibroblast function following the development of left ventricular failure. *J Mol Cell Cardiol*. 2006;40:474-483.

117. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res*. 1999;250:273-283.

118. Hautmann MB, Adam PJ, Owens GK. Similarities and differences in smooth muscle alpha-actin induction by TGF-beta in smooth muscle versus non-smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1999;19:2049-2058.

119. Levick SP, Gardner JD, Holland M, Hauer-Jensen M, Janicki JS, Brower GL. Protection from adverse myocardial remodeling secondary to chronic volume overload in mast cell deficient rats. *J Mol Cell Cardiol*. 2008;45:56-61.

120. Kasner M, Westermann D, Lopez B, Gaub R, Escher F, Kuhl U, Schultheiss HP, Tschope C. Diastolic tissue doppler indexes correlate with the degree of collagen expression and cross-linking in heart failure and normal ejection fraction. *J Am Coll Cardiol*. 2011;57:977-985.

121. Jugdutt BI. Ventricular remodeling after infarction and the extracellular collagen matrix: When is enough enough? *Circulation*. 2003;108:1395-1403.

122. Roten L, Nemoto S, Simsic J, Coker ML, Rao V, Baicu S, Defreyte G, Soloway PJ, Zile MR, Spinale FG. Effects of gene deletion of the tissue inhibitor of the matrix metalloproteinase-type 1 (TIMP-1) on left ventricular geometry and function in mice. *J Mol Cell Cardiol*. 2000;32:109-120.

123. Fedak PW, Smookler DS, Kassiri Z, Ohno N, Leco KJ, Verma S, Mickle DA, Watson KL, Hojilla CV, Cruz W, Weisel RD, Li RK, Khokha R. TIMP-3 deficiency leads to dilated cardiomyopathy. *Circulation*. 2004;110:2401-2409.

124. Lovelock JD, Baker AH, Gao F, Dong JF, Bergeron AL, McPheat W, Sivasubramanian N, Mann DL. Heterogeneous effects of tissue inhibitors of matrix metalloproteinases on cardiac fibroblasts. *Am J Physiol Heart Circ Physiol*. 2005;288:H461-8.

125. Zannad F, Pitt B. Biomarkers of extracellular matrix turnover. *Heart Fail Clin*. 2009;5:589-599.

Chapter 3

The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy *in vitro*

Bo Lu
Hasan Mahmud
Alexander H. Maass
Bo Yu
Wiek H. van Gilst
Rudolf A. de Boer
Herman H.W. Silljé

PLoS ONE. 2010 Sep 24;5(9):e12963.

Abstract

BI 2536 is a new anti-mitotic drug that targets polo-like kinase 1 (Plk1) and is currently under clinical development for cancer therapy. The effect of this drug on cancer cells has been extensively investigated, but effects on primary dividing cells and differentiated non-dividing cells are scarce. We have investigated the effects of this drug on primary neonatal rat cardiac fibroblasts and on differentiated cardiomyocytes and explored the possibility to use this drug to enrich differentiated cell populations *in vitro*. BI 2536 had a profound effect on cardiac fibroblast proliferation *in vitro* and arrested these cells in mitosis with an IC50 of about 43 nM. Similar results were observed with primary human cells (HUVEC, IC50=30 nM), whereas the cancer cell line HeLa was more sensitive (IC50 of 9 nM). Further analysis revealed that prolonged mitotic arrest resulted in cell death for about 40% of cardiac fibroblasts. The remaining cells showed an interphase morphology with mostly multi- and micro-nucleated nuclei. This indicates that a significant number of primary fibroblasts are able to escape BI 2536 induced mitotic arrest and apparently become aneuploid. No effects were observed on cardiomyocytes and hypertrophic response (growth) upon endothelin-1 and phenylephrine stimulation was normal in the presence of BI 2536. This indicates that BI 2536 has no adverse effects on terminally differentiated cells and still allows proliferation independent growth induction in these cells. In conclusion, cardiomyocytes could be enriched using BI 2536, but the formation of aneuploidy in proliferating cells, most likely limits this *in vitro* application and does not allow its use in putative cell based therapies.

Introduction

Contamination with proliferating cells is often a serious problem in cell culture studies investigating differentiated or quiescent cell populations, since the former can easily overgrow the cell type of interest. Examples include cardiomyocyte and neurological cell research, while also in the field of stem cell differentiation for cell therapies this represents a common problem. To remove proliferating cells from differentiated cell populations, nucleotide analogues are often used like bromodeoxyuridine (BrdU) and arabinoside [1, 2], which are incorporated in the DNA of proliferating cells resulting in DNA damage check-point activation and cell cycle arrest. Since these drugs affect the genetic code they cannot be used in any subsequent therapy. Moreover, these analogues are also incorporated in mitochondrial DNA and can interfere with mitochondrial biogenesis of the differentiated cell population. Other methods, like FACS analysis often require specific antibodies and the throughput is often limited. With the development of more specific anti-cancer drugs, we decided to investigate the potential of the polo-like kinase 1 (Plk1) inhibitor BI 2536 [3, 4] as a potential drug to eliminate proliferating cells from *in vitro* cultures containing terminally differentiated cardiomyocytes.

Plk1 is a mitotic kinase, which is highly expressed in proliferating cells only during the G2 and M phase of the cell cycle. It has specific roles during mitotic progression, including centrosome maturation, spindle assembly, chromosome segregation and cytokinesis [5, 6]. Plk1 consist of two domains, a C-terminal catalytic kinase domain and a N-terminal polo-box-domain (PBD), which recognizes specific phosphorylated targeting sequences [7, 8] and is essential for its specific localisation and interaction with some of its substrates [7, 9, 10]. Micro-injection of Plk1 antibodies and siRNA based studies targeting Plk1 have shown the essential role of Plk1 in mitotic progression in cancer cells [11-15]. These studies revealed that functional interference with Plk1 resulted in a mitotic arrest with condensed chromosomes, monopolar spindles and non-matured centrosomes. Recent studies with small molecules targeting Plk1, have confirmed these effects, and moreover revealed late stage mitotic functions for Plk1 [3, 16]. Following prolonged mitotic arrest, cell death (apoptosis) is induced in Plk1-inhibited cancer cells and hence Plk1 has been proposed to be a promising anti-cancer target [4, 17].

Despite the large body of evidence in cancer cells, the role of Plk1 in primary cells has only been poorly investigated and conflicting results have been published. In primary fibroblasts, Plk1 antibody microinjection was shown to arrest these cells with a G2-like phenotype, in contrast to the mitotic arrest in cancer cells [11]. G2-phase functions for Plk1, including recovery from DNA damage checkpoints, are supported by several studies [18-21].

Nevertheless, the role of Plk1 in G2-M phase transition in mammalian cells under normal conditions is still under debate [5]. Also siRNA studies showed different results in normal cells, as compared to cancer cells. In direct comparisons normal cells did not appear to be affected by Plk1 depletion, whereas cancer cells arrested in mitosis followed by cell death [12,13]. Together with the facts that numerous cancer cells show increased Plk1 expression, these results further supported that Plk1 could be a specific anti-cancer target [17].

BI 2536 is currently the most intensively investigated Plk1 inhibitor [22]. BI 2536 is a small molecule inhibiting Plk1 at subnanomolar concentrations in vitro (0.83 nM) and is equipotently active against human, mouse and rat Plk1 [4]. It was shown over 1000 fold more selective towards Plk1 as compared to 63 other kinases and only some activity against the closely related kinases Plk2 and Plk3 were reported [3]. BI 2536 has been tested on numerous human cancer cell lines *in vitro*, and tumor Xenograft models suggested prominent anti-cancer activity [3]. Several phase I and phase II trials have been performed [22-24], but its potential as an anti-cancer target still has to be established. The latest phase II trial study on five different cancer types was rather disappointing, showing only limited anti-tumor activity [24].

Here we tested the potential *in vitro* application of this drug by studying the elimination of proliferating cells from cultures containing primary differentiated cells. We used a commonly used system in cardiac research consisting of partially purified primary differentiated neonatal cardiomyocytes. We describe the effects of BI 2536 on these differentiated cells as well as on primary cardiac fibroblasts and show detrimental effects on primary proliferating cells.

Methods

Reagents and chemicals

Cell culture medium and serum were obtained from Lonza Benelux B.V. (Breda, The Netherlands) and penicillin-streptomycin and trypsin were from Invitrogen (The Netherlands). Mouse monoclonal anti-α-actinin antibody EA53 and mouse monoclonal anti-α-tubulin (B-5-1-2) antibody were from Sigma-Aldrich Chemie B.V., rabbit polyclonal anti-troponin (ab9332) was from Abcam (Cambridge, MA, USA), FITC labelled anti-phospho-Histone H3 (Ser10) antibody was from Cell Signaling. Antibody Dylight 649 labelling kit was from Pierce Biotechnology (Rockford, IL, USA) BI 2536 was purchased from Axon Medchem (Groningen, The Netherlands) and dissolved in DMSO at a concentration of 100 µM. L-[4,5-^{3}H]Leucine (37 MBq/ml; 5.85 TBq/mmol) was from GE Healthcare Europe (Diemen, Belgium) and Ultima Gold XR scintillation liquid was from Perkin Elmer (Groningen, The Netherlands). Unless

otherwise stated all chemicals were purchased at the highest possible grade from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

Neonatal rat cardiac myocyte and fibroblast isolation

Neonatal rat ventricle myocytes were isolated from the cardiac ventricles of 1-3 days old Sprague-Dawley pups. For isolation we followed a previously described protocol [1,25]. In short, hearts were removed from the thoracic cavity and placed in a tube containing cold CBFHH solution (137 mM NaCl, 5.36 mM KCl, 0.81 mM $MgSO_4$, 5.55 mM dextrose, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 20 mM HEPES, pH 7.4). Ventricles were separated from other tissue using scissors and cut into several pieces. Subsequently cardiomyocytes and fibroblasts were detached from the extracellular matrix by repeated incubation in CBFHH, supplemented with 2 mg/ml trypsin and 20 µg/ml DNase. Cells were collected by centrifugation and tissue clumps were removed by filtration. Subsequently, cells were preplated in cell culture dishes in 50 ml MEM medium with 5% FCS for 45 min. During this period most non-cardiomyocyte cells (mainly fibroblasts) attached to the dish, whereas cardiomyocytes remained in solution. The cardiomyocytes were subsequently transferred to separate tissue culture dishes and allowed to attach. Both fibroblasts and cardiomyocytes were subsequently cultured in DMEM medium containing 10% FCS.

All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen (Approval ID: DEC 5495).

Cell culture

Neonatal rat primary cardiac fibroblasts and cardiomyocytes and HeLa (S3) were cultured at 37° C under 5% CO_2 in DMEM medium, supplemented with 10% FCS and penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively). BI 2536 was added to a final concentration of 100 nM, unless otherwise stated. Corresponding control cultures received an equal volume of solvent (DMSO). Primary human umbilical vein endothelial cells (HUVEC) were obtained from the Endothelial Cell Facility (University Medical Center Groningen, The Netherlands). HUVEC were isolated from two umbilical cords and prepared as previously described [26, 27]. Cells were cultured on 1% pre-coated gelatin plates (Greiner Bio-One) at 37°C under 5% CO_2 in RPMI 1640 medium was supplemented with 20% FCS, 2 mmol/L L-Glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml endothelial cell growth factor extracted from bovine brain. HUVEC experiments were conducted with cell passage numbers between 2 and 6.

FACS analysis

For FACS analysis attached cells were detached by trypsin treatment and collected by centrifugation together with free floating cells in the medium. After a PBS wash cells were fixed in 4% paraformaldehyde for 10 min at 4°C. Cells were subsequently washed with PBS and stored at 4° C in PBS until all samples from one experiment were collected. Subsequently cells were treated with ice cold PBS+0.1% tritonX100 for 5 min and washed again with PBS. Cells were subsequently incubated in 100 µl PBS+1% BSA containing the indicated antibodies and propridium iodide (1 µg/ml) for 30 min at room temperature (RT). FITC labeled anti-phospho-Histone H3 (Ser10) antibody was used at a 1:200 dilution. Anti α-actinin antibody was first labelled with DyLight 649, according to the manufacturer instructions (Pierce Biotechnology, Rockford, IL, USA). This labelled antibody was then used at a 1:100 dilution. FACS analysis was performed on a FACS calibur (Becton Dickinson) and data were analysed with WinMDI2.9.

Quantitative Real-Time PCR

Relative expression of ANP, Troponin and Periostin genes was determined by quantitative PCR (qPCR). Gene expression was determined by correcting samples for reference gene values (cyclophilin A), and values were expressed relative to the control group per experiment. Primer sequences were: Troponin T-1, cagaagaggttggtcctgatgaa; Troponin T-2, gcaccaagttgggcatgaa; Periostin-1, gatccacggagaaccagtc; Periostin-2, cccacctcctgtggaaatc; Cyclophilin-1, cctcataccagcgacgattc; Cyclophilin-2, atgtggaggagtctcacttc.

Microscopy

For cardiomyocytes, coverslips were coated with 1 µg/cm^2 laminin (Millipore) for at least three hours. For fibroblasts non-coated coverslips were used and HUVEC cells were grown on coverslips coated with poly-lysine and 1% gelatin. Cells were grown on coverslips for the indicated time period and subsequently washed with PBS and fixed in paraformaldehyde solution for 10 min at RT. Cells were then permeabilised with ice cold PBS+0.5% Triton X100 for 5 min and then washed with PBS. Antibody incubations were performed in PBS+1%BSA for 1 hour at RT. Primary antibodies used were mouse monoclonal anti-α-tubulin and rabbit anti-troponin. Secondary antibodies were: goat anti-mouse Alexa555 (2 µg/ml) and goat anti-rabbit Alexa488 (2 µg/ml) and nuclei were stained with TO-PRO-3 (Invitrogen-Molecular Probes). Normal overview pictures and movies were made on a Leica DMIL microscope equipped with a Leica camera. Confocal images were made on a Leica SP2 AOBS confocal

microscope. All images were processed with ImageJ 1.43M (NIH, USA) and Adobe Photoshop 7.01.

[3H]-Leucine incorporation assay

Cells were grown in 12-well plates and subsequently starved for 24 hours in DMEM containing starvation medium lacking FCS. As described previously [28], L-[4,5-^{3}H]Leucine was added to all wells and cells were cultured for an additional 20 hours either in the presence or absence of 10 nM endothelin-1 (ET-1) or 50µM phenylephrine (PE). Subsequently, cells were washed twice with 1 ml PBS, followed by incubation for 1 hour at 4° C in 1 ml cold 5% TCA was. After one wash with TCA solution and one wash with PBS, proteins were solubilised with 0.5 ml 0.5M NaOH for 1 hour at RT and transferred to scintillation tubes containing 5 ml Ultima Gold XR scintillation liquid. The amount of radioactivity (CPM) was determined by a LS6500 Beckman Coulter scintillation counter.

Results

BI 2536 treatment of primary cardiomyocyte cultures

Primary cardiomyocytes were isolated from neonatal rat hearts and were cultured for three days in the presence of serum in order to adapt to the medium and allow any cardiomyocytes that have not reached end-stage differentiation to fulfil a last round of division. Subsequently, cardiomyocyte cultures were treated with the Plk1 inhibitor BI 2536 or were left untreated. As shown in Figure 1A, addition of 100 nM BI 2536, a concentration shown to arrest most cell lines maximally [4], resulted in a small increase in rounded up cells within 24 hours, indicative for a mitotic arrest. Further analysis by immunofluorescence microscopy confirmed that these rounded cells were in mitosis and contained predominantly monopolar spindles with condensed mitotic chromosomes (Figure 1B and data not shown). This is similar to what has been shown for cancer cell lines. Co-staining with troponin T, a cardiomyocyte specific marker revealed that the arrested cells were not cardiomyocytes and cardiomyocytes all showed normal interphase morphology (Figure 1B). To further confirm this, FACS analysis was performed using phospho-Histone H3 as a mitotic marker and α-actinin as a cardiomyocyte marker. In the control culture almost all cells were phospho-Histone H3 negative, and hence in interphase Figure 1C-D. Moreover, a significant proportion of cells were α-actinin negative, indicating the presence of non-cardiomyocytes. This relative high level of these cells is most likely a result of the three days cultivation without inhibitors. Importantly, after 24 hours BI 2536 treatment a significant portion of cells became phospho-

Histone H3 positive and these were almost all α-actinin negative (Figure 1C-D). This shows that cardiomyocytes were not sensitive to BI 2536 and that predominantly the non-cardiomyocyte cells were targeted by BI 2536.

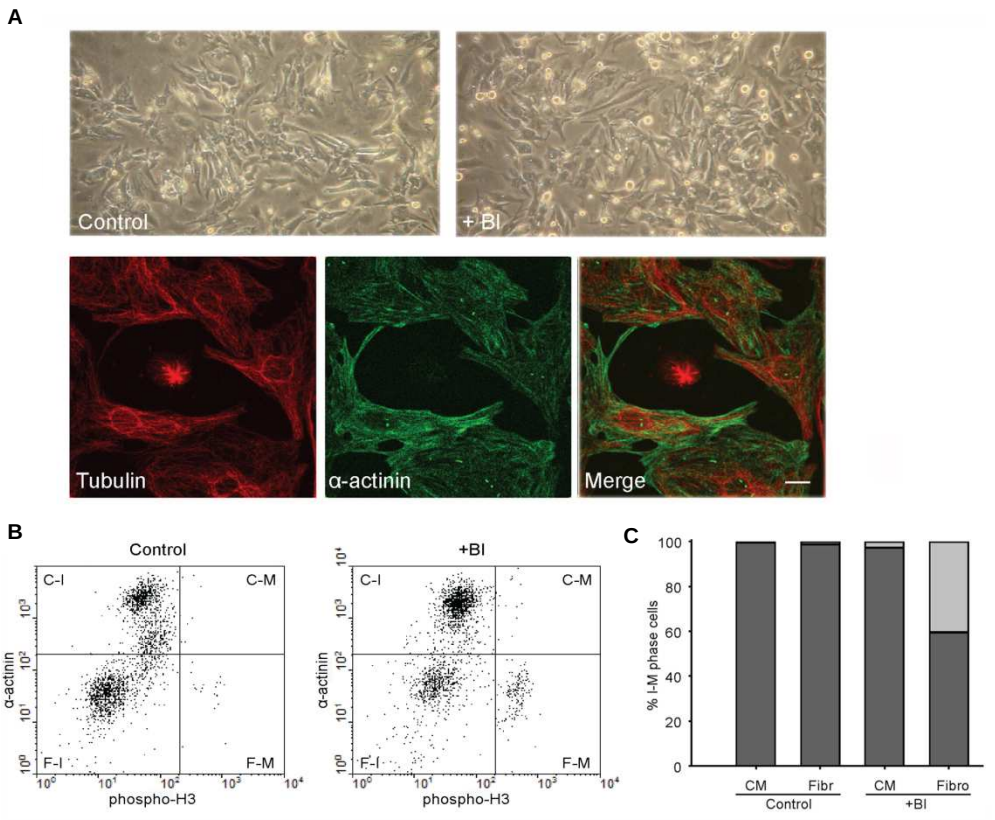


Figure 1. Effect of BI 2536 on neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were cultured for three days before 100 nM BI 2536, or DMSO as control, was added. A. Light microscopy pictures of control and 24 hours BI 2536 treated cultures. B. Immunofluorescence microscopy pictures of BI 2536 treated cells stained with anti-troponinT (red) and anti-α-tubulin (green). In the middle a mitotic cell is shown with monopolar spindle devoid of troponin T staining. Scale bar is 10 μM. C. FACS analysis of 24 hours treated and untreated cells stained with anti-α-actinin and anti-phospho-Histone H3. Four regions are selected and marked as follow: C-I = cardiomyocytes in interphase, C-M = cardiomyocytes in mitosis, F-I = fibroblasts in interphase, F-M = fibroblasts in mitosis. D. Quantification of FACS profiles of control and 24 hours BI 2536 (BI) treated cells stained like in C. Dark grey bars indicate the percentage interphase cells and light grey bars indicate the percentage mitotic cells. CM = cardiomyocyte, Fibro = fibroblast.

BI 2536 does not affect cardiomyocyte function and cellular growth (hypertrophy)

Next we addressed if cardiomyocyte function could be affected by BI 2536. We first examined the beating frequency of these cells by video imaging (data not shown). The beating frequency in BI 2536 treated cultures was similar to control cultures. Also β-adrenergic stimulation by isoproteronol was still normal in BI 2536 treated cells (data not shown). Even after two weeks incubation cardiomyocyte beating was still evident, indicating that BI 2536 does not show apparent interference with this major cardiomyocyte function.

Primary neonatal cardiomyocyte cultures are predominantly used as a model for cardiac hypertrophy. After two days culture of cardiomyocytes cells were treated with or without BI 2536 for 24 hours followed with a similar incubation in starvation medium. Thereafter cells were treated with the hypertrophy inducing stimuli endothelin-1 (ET-1) or phenylephrine (PE) in the presence of [^{3}H]Leucine. After 24 hours cells were harvested and the amount of [^{3}H]Leucine incorporation into protein was determined. As shown in Figure 2A ET-1 strongly increased [^{3}H]Leucine incorporation in cardiomyocytes and similar results were observed with PE (data not shown). This effect was even stronger in the presence of BI 2536,

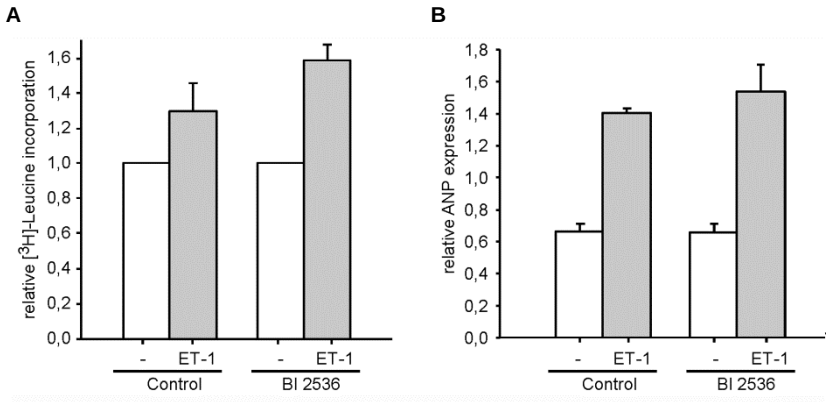


Figure 2. Effect of BI 2536 on hypertrophy induction in neonatal rat cardiomyocytes

Cardiomyocyte cultures were grown as before with or without 100 nM BI 2536 and subsequently serum starved in the presence or absence of BI 2536. A. Effect of treatment with the hypertrophy inducing hormone ET-1 on cellular growth ([^{3}H]-Leucine incorporation) in the presence or absence of BI. [^{3}H]-Leucine incorporation into cellular proteins was determined by liquid scintillation counting. Values are relative to the respective non-treated cultures and error bars depict standard deviations (n=3). B. Analysis of ANP gene expression by real-time PCR in cultures treated with or without ET-1 in the presence or absence of BI 2536. Levels are corrected for expression of the cyclophilin A housekeeping gene. Error bars depict standard deviations (n=3).

indicating that BI 2536 did not interfere with growth induction. The apparent stronger effect in the BI 2536 cultures, is a result of decreased fibroblast numbers in these cultures (see also below and Figure 3). To further corroborate this we also investigated ANP gene expression, which is a marker for the hypertrophic response and is expressed in cardiomyocytes only. As shown in Figure 2B, ANP expression was similarly up regulated in ET-1 treated cell cultures with and without BI 2536. Together, these results show that BI 2536 does not affect proliferation independent growth inducing responses.

Not all non-cardiomyocytes can be removed by BI 2536 treatment

The above results showed no apparent interference of cardiomyocytes function in the presence of BI 2536. Next we wanted to know if proliferating cells (>95% fibroblasts) in these cultures could be reduced by BI 2536 treatment. We included a mitotic shake-off to remove most mitotic cells before FACS analysis. As shown in Figure 3A, 24 hours BI 2536 treatment could already significantly reduce fibroblasts, resulting in a concomitant relative increase in the percentage of cardiomyocytes. This relative increase was further confirmed by the increase of troponin T expression, a cardiomyocytes specific marker, in these cultures (Figure 3B). Conversely expression of the fibroblast marker periostin decreased in these

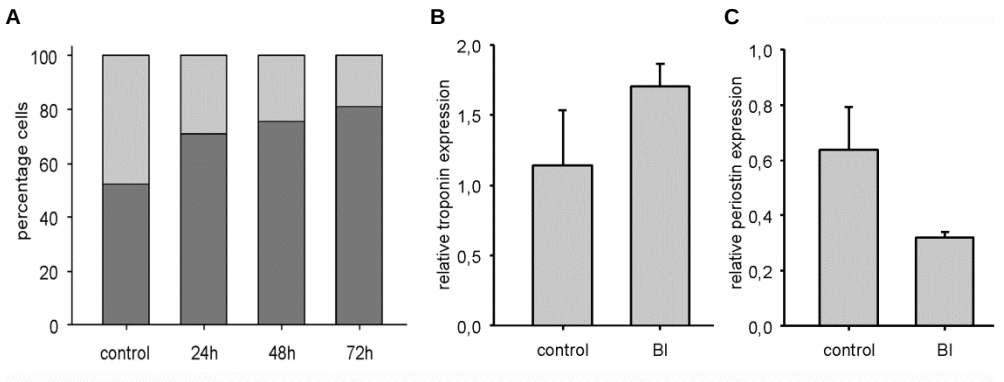


Figure 3. BI 2536 reduces fibroblast numbers in cardiomyocyte cultures.
Cardiomyocyte cultures were grown as before with or without 100 nM BI 2536. A. The percentage cardiomyocytes and cardiac fibroblasts were determined by FACS analysis, using anti-α-actinin staining. Before harvest mitotic cells were removed by shake off. Light gray bars indicate fibroblasts, dark grey bars show the percentage cardiomyocytes. B. After 24 hours treatment RNA was isolated from cells and real time PCR was performed with primers against cardiac troponin T to determine the expression level of this cardiomyocyte specific gene, providing an indirect measure for the number of cardiomyocytes. Error bars depict standard deviations (n=3). C. Same as in B, but with primers against periostin, a marker for cardiac fibroblasts.

cultures. This further confirmed that only non-cardiomyocytes, predominantly fibroblasts, were affected in these cultures. As shown in Figure 3A, prolonged incubation with BI could further lower the percentage of fibroblasts in these cultures. Nevertheless, even after 72 hours, still about 20% non-cardiomyocytes were present as determined by FACS analysis. This is surprising since these cells can perform 2-3 rounds of cell division within this time period (cycling time 28 hours). This suggests that not all proliferating cells are properly targeted by BI 2536.

BI 2536 induces mitotic arrest of primary cardiac fibroblasts

The contaminating cells in our cardiomyocyte cultures consisted predominantly of cardiac fibroblasts (>95%, data not shown). These cells are easily enriched by preplating and were subsequently used to test the effect of BI 2536 specifically on these primary proliferating cells. Since not all cells appeared to arrest with BI 2536 (Figure 3A), we analysed the concentration dependency. Using FACS analysis we determined the number of cells that arrested within 24 hours treatment with different BI 2536 concentrations. This dose response curve revealed that primary fibroblast arrest was maximal at a dose of 100 nM and the IC50 value was approximately 43 nM (Figure 4A). Not all cells did, however, arrest and a maximum of approximately 50% was observed at concentration of 100 nM and higher. The arrest of cardiac fibroblasts after 24 hours treatment with 100 nM BI 2536 was also clearly evident by the strong increase of rounded up cells (Figure 4B). Immunofluorescence microscopy confirmed the typical monopolar arrest of these cells, in contrast to the mostly bipolar mitotic cells in the control culture (Figure 4B). With HeLa cells an IC50 of only 9 nM was observed (Figure 4A), confirming previous results with these cells, and the maximal percentage of arrested cells was around 63%. At these low concentrations no effects were observed on neonatal rat cardiac fibroblast, showing that these cells are less sensitive. We also tested human primary HUVEC cells which arrested with an (IC50 of 30 nM), indicating that different cell types show different sensitivities.

BI 2536 temporarily arrests primary cardiac fibroblasts

To further corroborate the effect on primary fibroblasts, we also analysed the effect of BI 2536 in time. As shown in Figure 5A, 24 hours after treatment with BI 2536 a clear mitotic population (phospho-Histone H3 positive) is present, which is absent in the control culture. In time, however, this population is diminishing, and a population of dead, low propidium iodide stained cells became apparent. Prolonged culturing of control cells did only provide a small increase in dead cells. Quantification of FACS experiments (Figure 5B) revealed that after 24

hours about 41.5% of the cells were in mitosis and that 6.5% cells were dead. After 24 hours this has almost inverted with an average of 4.3% mitotic cells and 40.1% dead cells. Importantly, at this stage still about 55.1% of interphase cells were present. These cells were investigated by immunofluorescence microscopy and it turned out that more than 90% of these cells had multi- or micro-nucleated nuclei (Figure 5C), a clear sign of aberrant mitotic progression. We did not observe this micro-nucleation in cardiomyocytes treated with BI 2536 (data not shown), which further supports that this only occurred in proliferating cells, most likely due to an escape of the BI 2536 induced mitotic arrest. This also clarifies why BI 2536 treatment does not fully reduce the number of proliferating cells in cardiomyocyte cultures.

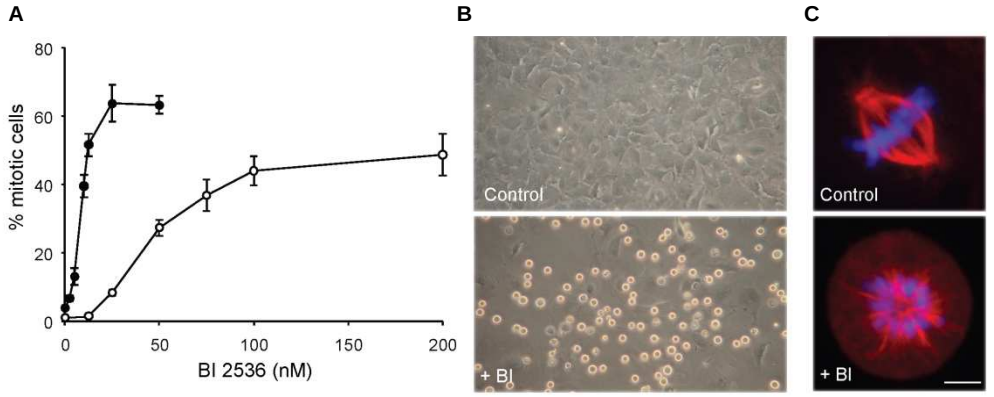


Figure 4. Effect of BI 2536 on rat cardiac fibroblasts

Rat cardiac fibroblasts were cultured for two days before the indicated doses of BI 2536 or DMSO as a control were added. HeLa cells were cultured under identical conditions. A. Dose response curve of primary cardiac fibroblasts and HeLa cells. Cells were cultured for 24 hours with BI 2536 and subsequently fixed and stained with anti-phospho-Histone H3, as a mitotic marker, and then quantified by FACS analysis. Closed circles, HeLa cells; open circles, cardiac fibroblasts. B. Light microcopy pictures of control and 24 hours BI 2536 treated cardiac fibroblast cultures. C. Immunofluorescence microscopy pictures of same cells as in B, but stained with anti-α-tubulin (green) and the DNA stain TO-PRO-3 (blue). Scale bar is 10 μM.

Discussion

In this paper we describe the effect of BI 2536 on primary cardiac fibroblasts and cardiomyocytes. We showed herein that BI 2536 does not generate adverse effects in cardiomyocytes, but does profoundly affect primary fibroblast. In particular, fibroblasts were

arrested in mitosis with monopolar spindles and died upon prolonged arrest. Nevertheless part of the cells escaped mitotic arrest, as was evident from the large number of multi- and micro-nucleated cells. Since aneuploidy is a hallmark of most solid cancers, this could be a

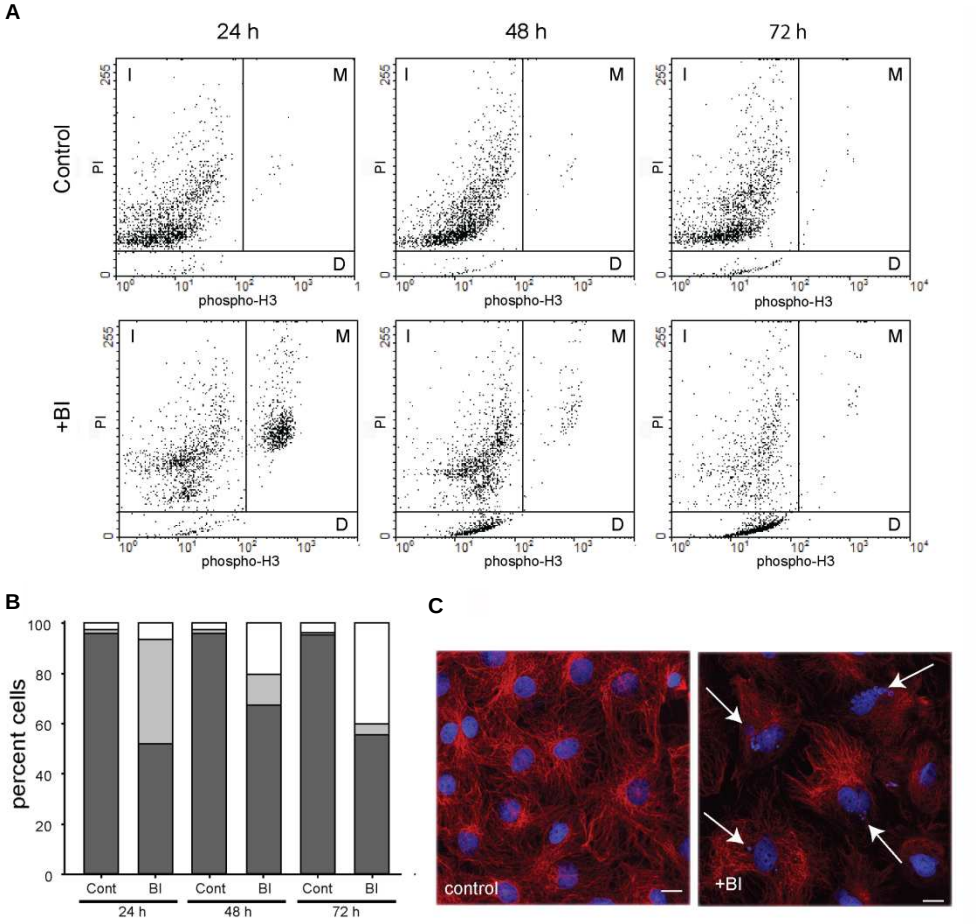


Figure 5. BI 2536 temporarily arrests cardiac fibroblasts

Rat cardiac fibroblasts were cultured for two days before 100 nM BI 2536 or DMSO as control were added. Cells were subsequently analysed at different time points. A. FACS analysis of cells stained with anti-phospho-Histone H3 and propidium iodide. In the upper panel the control cells are shown and in the lower panel the BI 2536 treated cells. The different regions are marked as follow: I is interphase, M is mitosis and D denotes dead cells. B. Quantification of FACS profiles, as shown in A (n=3). Dark grey bar depict interphase cells, light grey bar depicts mitotic cells and white bar depicts dead cells. C. Immunofluorescence pictures of remaining interphase cells after 24 hours culture in the presence or absence of BI 2536. Cells were stained with anti-α-tubulin (green) and the DNA stain TO-PRO-3 (blue). Control cells are shown in the upper panel. Scale bar is 10 μM.

potential concern for its use and will limit the potential of Plk1 inhibitors for enrichment of differentiated cells for cell therapy. As far as we know, this is the first analysis of the effects of BI 2536 on both primary and differentiated cells.

BI 2536 did not appear to affect the function of differentiated cells, as tested here using neonatal cardiomyocytes. No apparent morphological changes were observed and cardiomyocyte beating continued in the presence of BI 2536. Even after 4 weeks incubation with BI 2536 these cells continued normal beating (data not shown). Despite the fact that these cells cannot proliferate anymore, cardiomyocytes can still increase in size, and cardiomyocyte hypertrophy is one of the hallmarks of heart failure [29, 30]. We therefore also evaluated the effect of BI 2536 on the hypertrophic response to phenylephrine and endothelin-1 *in vitro*. As shown here cardiomyocyte hypertrophy could be induced in the presence of BI 2536, and even appeared more pronounced than in the absence of BI 2536. The latter was most likely due to the reduction in fibroblasts in these BI 2536 treated cultures, resulting in lower background values. Thus, BI 2536 specifically blocks proliferation, but does not seem to affect proliferation-independent hypertrophic cell growth of cardiomyocytes.

Our results with BI 2536 showed a clear mitotic arrest of primary rat fibroblast with monopolar spindles, which is similar to the observed arrest of cancer cells. This is in contrast to anti-Plk1 microinjection study in human fibroblasts, which showed predominantly a G2 phase-like arrest phenotype in primary cells, despite a clear mitotic arrest in cancer (HeLa) cells [11]. Although we cannot exclude a possible G2 delay in these cells, the major phenotype is mitotic arrest. A similar observation was made with primary human (HUVEC) cells, indicating that this is not due to species differences (data not shown). Plk1 siRNA based studies have suggested that Plk1 depletion did not affect normal cells or was much less effective in normal cells, as compared to cancer cells [12, 13]. Although higher concentrations of BI 2536 were required to arrest primary rat fibroblasts as compared to HeLa cels, we observed a clear mitotic arrest indicating that also in normal cells Plk1 is essential for proper mitotic progression. The absence of a mitotic phenotype with siRNA studies suggests, therefore, that depletion was not sufficient to generate this phenotype in normal cells.

Like in cancer cells arrested by Plk1 depletion or inhibition, we also observed cell death in primary cells following the mitotic arrest. In addition, however, we observed multi- and micro-nucleated cells in primary fibroblast populations treated with BI 2536. After 24 hours even more than 90% of the present interphase fibroblasts showed this multi- and micro-nucleation phenotype. This phenotype is a clear sign of abnormal mitotic progression, indicating that a substantial number of primary fibroblasts escaped the mitotic arrest. This effect has not been reported for cancer cells treated with BI 2536 [4]. This is surprising since it is often thought that, in contrast to normal cells, cancer cells might have a partially impaired

mitotic checkpoint, explaining chromosome instability in the latter [31]. Thus, it appears that cancer cells die more efficiently upon Plk1 inhibition, as compared to normal cells. The latter is in line with a recent study describing the effects of microtubule drugs on cancer and normal cells [32]. In this paper, the authors described that cancer cells are more susceptible to cell death upon mitotic arrest by the microtubule stabilizing agent taxol, as compared to normal cells. Our results suggest that this might be similar for Plk1 mediated mitotic arrests, suggesting that this is a general spindle checkpoint associated phenomenon. Although this is beneficial for the elimination of cancer cells, it begs the question what will happen with normal cells that have become aneuploid as a result of BI 2536 treatment. Since aneuploidy is a hallmark of most solid tumors [33] this could be a concern.

Altogether, our results show that Plk1 inhibition by BI 2536 treatment has no obvious effects on differentiated cardiomyocytes. It also, shows that whereas BI 2536 can inhibit proliferation it does not affect proliferation independent cell growth. Plk1 inhibition by BI 2536 resulted in mitotic arrest of primary fibroblast followed either by cell death or checkpoint slippage and aneuploidy. The latter will hamper the potential use of Plk1 inhibitors for the depletion of proliferating cells from differentiated cell cultures for cell therapy and might be a concern in general. Nevertheless, as shown here, it could be useful in cell cultures for *in vitro* applications.

Acknowledgements

We would like to thank Henk Moorlag for providing us with HUVEC cells and Silke Oberdorf-Maass for expert technical assistance and Klaas Sjollema of the UMCG Microscopy and Imaging Center (UMIC) for confocal microscopy assistance.

References

1. Maass AH, Buvoli M (2007) Cardiomyocyte preparation, culture, and gene transfer. *Methods Mol.Biol.* 366:321-330.

2. Ren D, Miller JD (2003) Primary cell culture of suprachiasmatic nucleus. *Brain Res.Bull.* 61, 547-553.

3. Lenart P, Petronczki M, Steegmaier M, Di Fiore B, Lipp JJ, et al. (2007) The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr.Biol.*17, 304-315.

4. Steegmaier M, Hoffmann M, Baum A, Lenart P, Petronczki M, et al (2007) BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. *Curr.Biol.* 17, 316-322.

5. Barr FA, Sillje HH, Nigg EA (2004) Polo-like kinases and the orchestration of cell division. *Nat.Rev.Mol.Cell Biol.* 5, 429-440.

6. van de Weerdt BC, Medema RH (2006) Polo-like kinases: a team in control of the division. *Cell Cycle.* 5, 853-864.

7. Elia EA, Cantley LC, Yaffe MB (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science.* 299, 1228-1231.

8. Elia EA, Rellos P, Haire LF, Chao JW, Ivins FJ, et al (2003) The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell.* 115, 83-95.

9. Hanisch A, Wehner A, Nigg EA, Sillje HH (2006) Different Plk1 functions show distinct dependencies on Polo-Box domain-mediated targeting. *Mol.Biol.Cell.* 17, 448-459.

10. Lowery DM, Clauser KR, Hjerrild M, Lim D, Alexander J, et al. (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. *EMBO J.* 26, 2262-2273.

11. Lane HA, Nigg EA(1996) Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes. *J.Cell Biol.* 135, 1701-1713.

12. Elez R, Piiper A, Kronenberger B, Kock M, Brendel M, et al. (2003) Tumor regression by combination antisense therapy against Plk1 and Bcl-2. *Oncogene.* 22, 69-80.

13. Liu X, Lei M, Erikson RL (2006) Normal cells, but not cancer cells, survive severe Plk1 depletion. *Mol.Cell Biol.* 26, 2093-2108.

14. an Vugt MA, van de Weerdt BC, Vader G, Janssen H, Calafat J, etal. (2004) Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. *J.Biol.Chem.* 279, 36841-36854.

15. Spankuch-Schmitt B, Bereiter-Hahn J, Kaufmann M, Strebhardt K (2002) Effect of RNA silencing of polo-like kinase-1 (PLK1) on apoptosis and spindle formation in human cancer cells. *J.Natl.Cancer Inst.* 94, 1863-1877.

16. A.Santamaria A, R.Neef R, U.Eberspacher U, K.Eis K, M.Husemann M, et al. (2007) Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis. *Mol.Biol.Cell.* 18, 4024-4036.

17. Strebhardt K, Ullrich A (2006). Targeting polo-like kinase 1 for cancer therapy. *Nat.Rev.Cancer.* 6, 321-330.

18. van Vugt MA, Bras A, Medema RH (2004) Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. *Mol.Cell.* 15, 799-811.

19. Kumagai A, Dunphy WG (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. *Science.* 273, 1377-1380.

20. Nakajima H, Toyoshima-Morimoto F, Taniguchi E, Nishida E (2003) Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J.Biol.Chem.* 278, 25277-25280.

21. Okano-Uchida T, Okumura E, Iwashita M, Yoshida H, Tachibana K, et al. (2003) Distinct regulators for Plk1 activation in starfish meiotic and early embryonic cycles. *EMBO J.* 22, 5633-5642.

22. Schoffski P (2009) Polo-like kinase (PLK) inhibitors in preclinical and early clinical development in oncology. *Oncologist.* 14, 559-570.

23. Mross K, Frost A, Steinbild S, Hedbom S, Rentschler J, et al. (2008) Phase I dose escalation and pharmacokinetic study of BI 2536, a novel Polo-like kinase 1 inhibitor, in patients with advanced solid tumors. *J.Clin.Oncol.* 26, 5511-5517.

24. Schoffski P, Blay JY, De Greve J, Brain E, Machiels JP, et al. (2010) Multicentric parallel phase II trial of the polo-like kinase 1 inhibitor BI 2536 in patients with advanced head and neck cancer, breast cancer, ovarian cancer, soft tissue sarcoma and melanoma. The first protocol of the European Organization for Research and Treatment of Cancer (EORTC) Network Of Core Institutes (NOCI). *Eur.J.Cancer.*

25. Maass A, Langer SJ, Oberdorf-Maass S, Bauer S, Neyses L, et al. (2003) Rational promoter selection for gene transfer into cardiac cells. *J.Mol.Cell Cardiol.* 35, 823-831.

26. Oeseburg H, de Boer RA, Buikema H, van der Harst P, van Gilst WH, et al. (2010) Glucagon-Like Peptide 1 Prevents Reactive Oxygen Species-Induced Endothelial Cell Senescence Through the Activation of Protein Kinase A. *Arterioscler.Thromb.Vasc.Biol.* 30, 1407-1414.

27. Schraa AJ, Kok RJ, Berendsen AD, Moorlag HE, Bos EJ, et al. (2002) Endothelial cells internalize and degrade RGD-modified proteins developed for tumor vasculature targeting. *J.Control Release.* 83, 241-251.

28. Maass A, Grohe C, Kubisch C, Wollnik B, Vetter H, et al. (1995) Hormonal induction of an immediate-early gene response in myogenic cell lines--a paradigm for heart growth. *Eur.Heart J.* 16 Suppl C:12-4., 12-14.

29. Frey N, Katus HA, Olson EN, Hill JA (2004) Hypertrophy of the heart: a new therapeutic target? *Circulation.* 109, 1580-1589.

30. Hill JA, Olson EN (2008) Cardiac plasticity. *N.Engl.J.Med.* 358, 1370-1380.

31. Kops GJ, Weaver BA, Cleveland DW (2005) On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat.Rev.Cancer.* 5, 773-785.

32. Brito DA, Rieder CL (2009) The ability to survive mitosis in the presence of microtubule poisons differs significantly between human nontransformed (RPE-1) and cancer (U2OS, HeLa) cells. *Cell Motil.Cytoskeleton.* 66, 437-447.

33. Rajagopalan H, Lengauer C (2004) Aneuploidy and cancer. *Nature.* 432, 338-341.

Chapter 4

Identification of hypertrophy and heart failure associated genes by combining *in vitro* and *in vivo* models

Bo Lu
Hongjuan Yu
Maarten Zwartbol
Willem P.T. Ruifrok
Wiek H. van Gilst
Rudolf A. de Boer
Herman H.W. Silljé

Physiol Genomics. Under revision.

Abstract

Heart failure (HF) is a complex disease involving multiple changes including cardiomyocyte hypertrophy (growth). Here we performed a set of screens in different HF and hypertrophy models to identify differentially expressed genes associated with HF and/or hypertrophy. Hypertensive Ren2 rats and animals with post-MI HF were used as *in vivo* HF models and neonatal rat cardiomyocytes treated with hypertrophy inducing hormones phenylephrine, endothelin-1 and isoproterenol were used as *in vitro* models. This combined approach revealed a robust set of genes that were differentially expressed both *in vitro* and *in vivo*. This included known genes like NPPA (ANP), Xirp2 and FHL1, but also novel genes not previously associated with hypertrophy/HF. Amongst these are PTGIS, AKIP1 and Dhrs7c, which could constitute interesting targets for further investigations. We also identified a number of *in vivo* specific genes and these appeared to be enriched for fibrosis, wounding and stress responses. Therefore a number of novel genes within this *in vivo* specific list could be related to fibroblasts or other non-cardiomyocytes present in the heart. We also observed strong differences between the two HF rat models. For example CA3 and WISP2 were strongly upregulated in post-MI HF, but not in Ren2 rats, indicative for etiology specific differences. Together these results show that combining multiple models, both *in vivo* and *in vitro*, can provide a robust set of hypertrophy/HF associated genes. Moreover it provides insight in the differences between the different etiology models and neurohormonal effects.

Introduction

Heart failure (HF) is often the end-result of a number of cardiovascular diseases, including myocardial infarction, hypertension and valve abnormalities. To maintain sufficient cardiac output under these conditions the heart adapts to these changes, a process called cardiac remodeling [13]. Cardiac fibrosis and hypertrophy are the two major processes driving cardiac remodeling.

The initiating stimuli for cardiac hypertrophy can be broadly separated into biomechanical or stretch-sensitive mechanisms and neurohormonal mechanisms [12]. The latter include amongst others catecholamines (adrenaline and noradrenaline) and endothelin-1, which bind to specific plasmamembrane receptors and activate intracellular signaling pathways [12]. Although these signaling pathways are inherently complex and involve significant cross-talk, the common outcome, besides hormone specific effects, is the induction of cardiomyocyte growth. This involves direct activation of protein synthesis and altered gene expression. A hallmark of cardiac hypertrophy is the activation of the so called fetal gene program, which includes the re-expression of a number of fetal genes [24]. Important examples are atrial natriuretic peptide (ANP, NPPA) and brain natriuretic peptide (BNP, NPPB), and the latter is commonly used as a HF plasma marker [16].

Numerous global gene expression studies have been performed on human or animal heart tissue to identify genes that are differentially expressed during HF. Usually gene expression is compared between opposing pairs, such as non-failing vs. failing hearts and once it is performed it is essential to narrow down the large candidate gene lists. The gene sets of different studies only partly overlap, because of diversity in disease severity, etiologies and microarray platforms [1]. Meta-analysis of gene expression studies has therefore identified some HF genes, including ANP, BNP, ACTC1 (α cardiac actin), POSTN (periostin), but also revealed that most genes were only found in a limited set of studies [1]. Gene array studies have also been performed on cultured primary neonatal rat cardiomyocytes [3, 5, 10, 14], which are used as a general *in vitro* cardiomyocyte hypertrophy model. Compared to *in vivo* studies it has the advantage that it involves a relatively pure cell population, that there are no systemic effects present in the background, and that conditions can be well controlled. However, *in vitro* observations do not necessarily reflect *in vivo* changes. Also these studies generated large candidate lists that were not easily comparable, because of different platforms, time durations and stimuli used [3, 5, 10, 14].

As far as we know, no direct comparisons have been made between hypertrophy/HF induced *in vitro* and *in vivo* gene expression changes. This combination could provide a more robust outcome and, moreover, might identify *in vitro* and *in vivo* differences.

We therefore treated neonatal rat ventricular cardiomyocytes (NRVCs) with hypertrophy stimulating agents phenylephrine (α-adrenergic), isoproterenol (β-adrenergic) and endothelin-1. In addition we used two different HF models, namely Ren2 rats that overexpress mouse renin generating hypertension and resulting in HF and we used a rat post-myocardial infarction (MI) model. Our results show overlap between the models and identified some potential novel hypertrophy/HF associated genes. In addition interesting differences between the different models and conditions were observed.

Materials and methods

Reagents and chemicals

Cell culture medium and serum were obtained from Lonza Benelux B.V. (Breda, The Netherlands), penicillin-streptomycin and trypsin were from Invitrogen (The Netherlands). L-[4,5-^{3}H]-Leucine (37 MBq/ml; 5.85 TBq/mmol) was from GE Healthcare Europe (Diemen, Belgium) and Ultima Gold XR scintillation liquid was from Perkin Elmer (Groningen, The Netherlands). Unless otherwise stated all chemicals were purchased at the highest possible grade from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

Cardiomyocyte isolation and culturing

NRVCs were isolated from the cardiac ventricles of one to three days old Sprague-Dawley (SD) pups after decapitation, as previously described [19]. In short, hearts were removed from the thoracic cavity and ventricular tissue fragments were repeatedly treated with 2 mg/ml trypsin to release the cells. To remove non-cardiomyocytes, isolated cells were preplated in cell culture dishes in 50 ml MEM medium with 5% FCS for 45 min. During this period, most non-cardiomyocytes (mainly fibroblasts) attached to the dish (Greiner Bio-One), whereas cardiomyocytes remained in solution. The cardiomyocytes were subsequently transferred to separate tissue culture dishes and allowed to attach. NRVCs were cultured at 37° C under 5% CO_2 in DMEM medium, supplemented with 10% FCS and penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively), as described previously [19]. For experiments in which cells were treated with hypertrophy inducing agents, cells were first serum starved for 24 hours. Cells were then treated 24 hours with either 50 µM phenylephrine (PE), 10 µM isoproterenol (Iso) or 10 nM endothelin-1 (ET-1).

Animal experiments

We used male TGR(mRen2)27 (Ren2) rats that were homozygous for the expression of the

mouse renin (Ren)-2 transgene (Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany) [7, 17]. SD rats were used as controls. Animals were fed *ad libitum*. Ren2 rats and control rats were sacrificed at 13 weeks of age [26]. In parallel, HF was induced in SD rats by permanent ligation of the left coronary artery to produce a MI [18]. Anesthetic agents used: isoflurane (5% induction, 2.5% maintenance with oxygen) and proper anesthesia was confirmed by reflex analysis and by heart rate monitoring. Analgesics used: buprenorphine 0.04 mg/kg, every 8 hours for 3 days. Control rats underwent a sham operation. Nine weeks after MI animals were sacrificed. All animals were sacrificed under general anesthesia, as described above, by heart removal. Myocardial tissues from all groups were snap frozen for molecular analyses. All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were submitted to, and approved by, the Committee for Animal Experiments of the University of Groningen.

Quantitative Real-Time PCR

Relative gene expression of ANP, cyclophilin A and GAPDH was determined by quantitative real time PCR (RT-PCR) on a Bio-Rad CFX384 real time system using SYBR Green dye, as described before [23]. Gene expression was determined by correcting for reference gene values (cyclophilin A or GAPDH), and the calculated values were expressed relative to the control group per experiment. Primer sequences of ANP and cyclophilin A have been described before [19]. GAPDH sequences are: GAPDH-forward-catcaagaaggtggtgaagc; GAPDH-reverse-accaccctgttgctgtag.

L-[4,5-^{3}H]-Leucine incorporation assay

Cardiomyocytes were grown in 12-well plates and subsequently starved for 24 hours in DMEM containing starvation medium lacking FCS. Subsequently, L-[4,5-^{3}H]-Leucine was added to all wells either in the presence or absence of 50 µM PE, 10 µM Iso or 10 nM ET-1. Cells were cultured for an additional 24 hours and radioactivity incorporation was determined as described before [19].

Microarray Sample Preparation and Analysis

Total RNA from the cells or tissue was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). RNA quality was checked using the Agilent 2100 Bioanalyzer™ and subsequently prepared for microarray analysis using the standard Illumina® TotalPrep™-96 RNA Amplification Protocol (Ambion, Inc.). Samples were exponentially amplified from a starting amount of 50 ng to a final amount of 1 µg purified biotin-labeled

cRNA using the Illumina® TotalPrep™-96 RNA Amplification Kit (Ambion, Inc.). This final cRNA was evaluated and the quality, concentration and size of the reaction productions were measured using the Agilent RNA 6000 Nano Kit (Agilent). Illumina RatRef-12 Beadchips were used for microarray analysis. Chips were scanned using the BeadXpress Reader™ (Illumina). Beadstudio™ Illumina was used to import the raw data and remove any background noise. Data was converted to standard format and exported for use in Agilent Genespring GX™ (version 11.0, Agilent). We used Genespring to perform quantile normalization on each individual well and further analysis of the data. The MIAME standard for microarray data was followed and all data were submitted to the CIBEX database (accession codes: CBX250 and CBX251).

Statistical analysis

All data are expressed as mean±SEM. The differences between two groups were determined by a Student's *t* test. Comparisons between more than two groups of data were assessed by One-Way ANOVA followed by Bonferroni's post-hoc test. A value of $P<0.05$ was considered significant.

Results

In vivo rat HF models for gene expression analysis

To identify genes that are differentially expressed during hypertrophy and HF development, we used a combined *in vitro/in vivo* gene array approach. For the *in vivo* analysis two different animal HF models were compared to their respective control groups. One group consisted of transgenic Ren2 rats (n=4), which overexpress mouse renin, resulting in hypertension and consequently HF development [7, 17]. These animals were compared to SD rats, their genetic wild type background (n=5). In the other rat model a MI was induced by permanent ligation of the left coronary. These animals were sacrificed nine weeks post-MI together with the sham operated animals (n=5, 4). As determined by the heart weight-to body weight (HW/BW) ratios, significant cardiac hypertrophy was observed in both the Ren2 rats (5.06±0.05 vs. 3.29±0.11 mg/g in the control, $p<0.05$) and also in the post-MI rats a clear increase (4.34±0.52 vs. 3.65±0.40 mg/g in the control) was apparent. Moreover, cardiac ANP expression, a marker for HF, was significantly increased in the Ren2 and post-MI rats (Figure 1A, 1B). Hemodynamic data further confirmed HF formation in these animals (data not shown).

In vitro cardiomyocyte hypertrophy models

The *in vitro* hypertrophy model consisted of cultures of primary isolated NRVCs , which were exposed to three different hypertrophy inducing agents (n=4 in each group, PE n=3). As determined by L-[4,5-^{3}H]-leucine incorporation (protein synthesis), treatment with PE and ET-1 generated a strong hypertrophic response, whereas Iso induced a modest increase (Figure 1C). A similar trend was observed for ANP gene expression, which was strongly elevated after PE and ET-1 treatment and only modestly elevated after Iso treatment (Figure 1D). Together, this shows that these treatments trigger hypertrophy and fetal gene expression in cardiomyocytes *in vitro*.

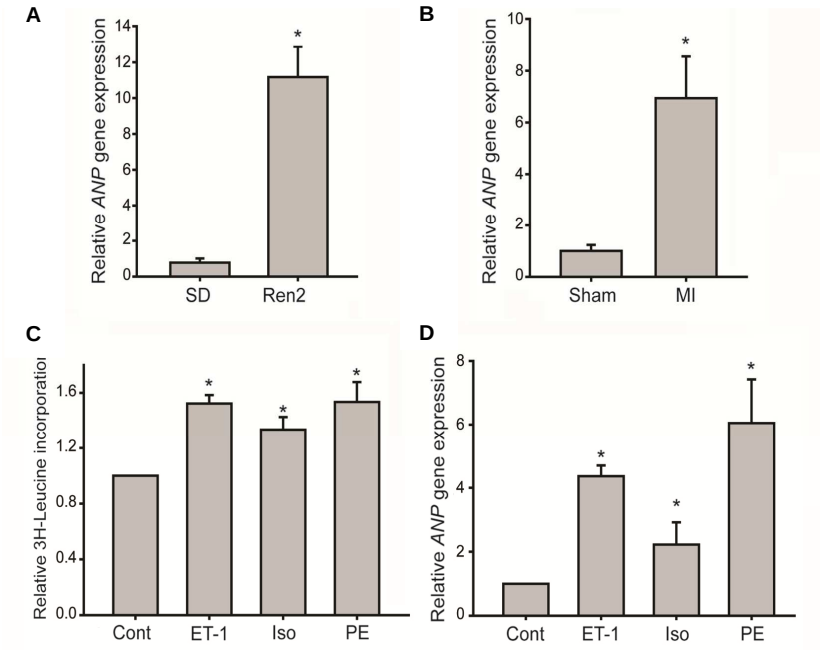


Figure 1. Confirmation of the *in vivo* and *in vitro* hypertrophy/HF models

A. RT-PCR on cDNA from Ren2 rats (n=5) with HF and as a control on SD rats (n=4 for each group). *ANP* expression is shown relative to *GAPDH* levels. B. RT-PCR on cDNA from post-MI HF and sham operated rats (n=4 for each group). *ANP* expression is shown relative to *GAPDH* levels. C. Primary neonatal rat cardiomyocytes were cultured for 24 hours in serum free medium and subsequently stimulated with PE, Iso or ET1. After 24 hours stimulation in the presence of L-[4,5-^{3}H]-Leucine the radioactive incorporation in cellular protein was analyzed by liquid scintillation counting. Values are relative to the respective non-treated cultures (n=4 for each group). D. *ANP* expression in the different cardiomyocyte cultures was determined by RT-PCR and corrected for *cyclophilin A* expression (n=4).

SD=Sprague Dawley rats, Ren2=TGRm(Ren2)27 rats, MI= myocardial infarction, cont=control, ET-1=endothelin-1, Iso=isoproterenol, PE=phenylephrine, ANP= atrial natriuretic peptide. Data is presented as mean±SEM.

Gene array analysis animal models

Whole genome gene expression in the cardiac animal samples and of the cardiomyocyte cultures was analyzed using Illumina RatRef-12 Beadchip arrays. Only those genes that showed a significant 1.5 fold change in gene expression ($p<0.05$) as compared to their respective control groups were selected. This yielded 159 differentially expressed genes in the Ren2 group and 254 differentially expressed genes in the MI group. Subsequently, the overlap in both HF groups was analyzed, yielding only a small set of 58 genes (16%) that were similarly up- or downregulated in both groups (Figure 2A). Since genes that are just above selection threshold in one group, might be missed in the other group this representation could give a biased view. Therefore we also analyzed whether the top 10 up- and downregulated genes in the Ren2 and MI groups showed a similar distribution. As can be depicted from Table 1 this is indeed the case. Within this top ten several common HF markers, including periostin (POSTN) [22] and ANP [16], were similarly upregulated in both animal groups. It is remarkable, however, that the two strongest upregulated genes in the MI group, CA3 (Carbonic anhydrase 3) and WISP2 (Wnt1 inducible signaling pathway protein 1), were not upregulated in the Ren2 group. *Vice versa* the two strongest upregulated genes in

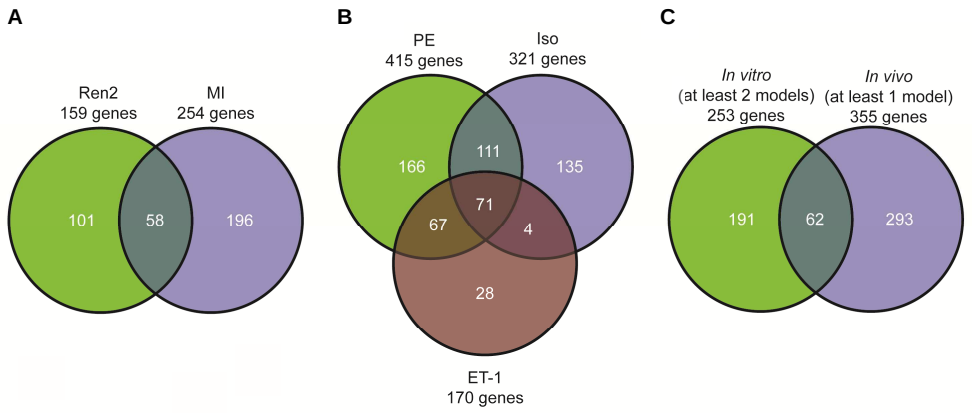


Figure 2. Venn diagrams of the number of differentially expressed genes identified in the different groups

A. Differential gene expression in different groups was determined as described in the text. A. Differentially expressed genes in Ren2 and post-MI animals as compared to their respective controls. B. Differentially expressed genes identified after stimulation for 24 hours with PE, Iso or ET-1. C. Differentially expressed genes present in at least in two *in vitro* models were compared with the differentially expressed genes present in at least one *in vitro* model. An overlap of 62 genes was identified.

Ren2= TGRm(Ren2)27 rats, MI=myocardial infarction, ET-1=endothelin-1, Iso=isoproterenol, PE=phenylephrine.

the Ren2 group, KCNE1 (Potassium Channel, voltage-gated, ISK related subfamily, member 1) and RGD1562305 (SBSN, Suprabasin), were not upregulated in the MI group. The full gene tables are presented in Supplemental gene list 1.

In vitro cardiomyocyte hypertrophy models

Whole genome gene expression of the hypertrophy induced cardiomyocyte cultures was compared to the untreated culture group. This revealed that 415, 170 and 321 genes were differentially expressed after PE, ET-1 and Iso treatment respectively ($p<0.05$, >1.5 fold difference) (Figure 2B). Thus both adrenergic stimuli (PE and Iso) gave a stronger hypertrophic effect than ET-1, even though hypertrophy development with Iso was less pronounced, as compared to ET-1 (Figure 1C). Comparison of these data sets showed a common overlap of 71 genes. A list of the 10 most upregulated genes present in at least two conditions is presented in Table 2. Most of the top ten genes were upregulated by all three stimuli and include known cardiac disease genes, like Xirp2, Scn3b and ACTC1. Although HF markers, like ANP and BNP, were not present in this top these are present in the full gene list (Supplemental gene list 2). It is remarkable that the strongest upregulated gene, PNOC (Prepronociceptin) was specific for PE and Iso stimulation. In addition, the potassium voltage gated channel, KCND2 was upregulated only in ET-1 treated cells and the aldoketo reductase gene AKR1B8 was specific for Iso stimulation. Most downregulated genes were specific for adrenergic stimulation (Iso and/or PE), including the strongly downregulated short chain dehydrogenase/reductase gene Dhrs7c. Only one gene of the top 10 genes was downregulated by all three stimuli, ANGPT2 (angiopoietin 2), a protein involved in vascular remodeling [9].

Comparison of the *in vitro* and *in vivo* data sets

To compare the *in vitro* data with the *in vivo* data all differentially *in vitro* expressed genes that were present in at least 2 conditions (253 genes) were compared with all differentially *in vivo* expressed genes (355 genes). This yielded a set of 62 genes differentially expressed in at least two *in vitro* and one *in vivo* model (Figure 2C). Within the group of 62 genes, established hypertrophy/HF markers like ANP and BNP and a promising new HF marker LGALS3 (galectin-3) were present [6, 8]. Based on the average fold change gene expression we made a list of the top 10 up- and downregulated genes found in at least 2 *in vitro* models and one *in vivo* model (Table 3). In this list, a number of known HF markers and cardiac disease genes are present, like ANP, XIRP2, CSRP2, FHL1, TIMP1 and LGALS3. However, it also includes novel upregulated genes like AKIP1 (also termed BCA3 or C11Orf17), PTGIS and downregulated genes, like DHRS7c (Table 3). We also like to note that within the set of

62 genes there are 9 genes that are regulated in an opposite manner *in vitro* as compared to *in vivo* (Supplemental gene list 3). We also identified a large number of genes that showed differential expression *in vitro*, but not *in vivo* and *vice versa* (Table 4).

Table 1. Top ten genes with highest up- or downregulation in at least one *in vivo* model (1.5 fold, p<0.05)

Gene	Genbank Id	Protein	Regulation	Ren2	MI
CA3	54232	carbonic anhydrase III	Up	-	47.71
WISP2	29576	WNT1 inducible signaling pathway protein 2	Up	-	28.79
LTBP2	59106	latent transforming growth factor beta binding protein 2	Up	8.64	24.93
KCNE1	25471	potassium voltage-gated channel, Isk-related family, member 1	Up	24.67	-
RGD1562305	292793	suprabasin	Up	16.09	-
THBS4	29220	thrombospondin 4	Up	-	14.15
POSTN	361945	periostin, osteoblast specific factor	Up	8.82	13.50
SDPR	316384	serum deprivation response	Up	11.17	-
PRG4	289104	proteoglycan 4	Up	-	10.88
ANP	24602	atrial natriuretic peptide	Up	9.39	5.96
RT1-A1	24973	RT1 class Ia, locus A1	Down	-20.52	-
RGD1561381	498340	similar to microsomal glutathione S-transferase 3	Down	-20.29	-
RPS9	81772	ribosomal protein S9	Down	-19.53	-
LOC498989	498989	similar to Ab2-143	Down	-13.85	-
RGD1564649	367102	similar to 40S ribosomal protein S9	Down	-11.94	-
USMG5	171069	up-regulated during skeletal muscle growth 5 homolog	Down	-11.78	-
VEGFB	89811	vascular endothelial growth factor B	Down	-9.52	-
MRPL17	171061	mitochondrial ribosomal protein L17	Down	-7.39	-
SLC3a1	29484	solute carrier family 3, member 1	Down	-4.24	-
RPS9	81772	ribosomal protein S9	Down	-4.06	-

– means absence of up/or down regulation. Ren2 (n=4), SD=Sprague Dawley (n=5), MI=myocardial infarction (n=5) and sham (n=4).

Table 2. Top ten genes with highest up- or downregulation in at least one *in vitro* model (>1.5 fold, p<0.05)

Gene	Genbank Id	Protein	Regulation	ET-1	Iso	PE
PNOC	25516	prepronociceptin	Up	-	28.35	42.36
WFDC1	171112	WAP four-disulfide core domain 1	Up	3.89	5.30	11.05
Scn3b	245956	sodium channel, voltage-gated, type III, beta	Up	4.37	4.98	6.82
Xirp2	497771	xin actin-binding repeat containing 2	Up	4.14	3.29	5.85
INHA	24504	inhibin alpha	Up	2.13	4.48	5.62
ACTC1	29275	actin, alpha, cardiac muscle 1	Up	3.37	3.37	5.05
RGD1560242	499334	similar to RIKEN cDNA 1700028P14	Up	1.83	3.69	4.84
AKR1B8	286921	aldoketo reductase family 1, member B8	Up	-	4.55	-
KCND2	65180	potassium voltage-gated channel, Shal-related subfamily, member 2	Up	4.31	-	-
RND1	362993	Rho family GTPase 1	Up	2.06	3.91	4.25
Dhrs7c	287411	dehydrogenase/reductase (SDR family) member 7C	Down	-	-20.18	-4.12
LOC501066	501066	WITHDRAWN	Down	-	-6.31	-
CXCL11	305263	chemokine (C-X-C motif) ligand 11	Down	-	-5.56	-3.17
Asb11	302666	ankyrin repeat and SOCS box containing 11	Down	-	-4.81	-2.04
PTPRR	94202	protein tyrosine phosphatase, receptor type, R	Down	-	-4,79	-4,58
Angpt2	89805	angiopoietin 2	Down	-1.92	-4,01	-4,66
CITED1	64466	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	Down	-	-3.75	-1.96
V-ATPase S1	361875	ATPase, H+ transporting, lysosomal accessory protein 1	Down	-	-3.66	-
RXRG	83574	retinoid X receptor gamma	Down	-	-3.47	-3.12
CXCL10	245920	chemokine (C-X-C motif) ligand 10	Down	-	-3.30	-

– means absence of up/or down regulation. Control (n=4), ET-1=endothelin-1 (n=4), Iso=isoproterenol (n=4) and PE =phenylephrine (n=3).

Gene ontology analysis

Finally, we performed gene ontology (GO) analysis using Genetrail with the different data sets. In most data sets no specific enrichment of gene clusters with particular functions was obtained, but for the *in vitro* and *in vivo* specific data sets some highly significant scores were

Table 3. Top ten genes with highest up- or downregulation in at least two *in vitro* models and at least one *in vivo* model (1.5 fold, p<0.05)

Gene	Genbank Id	Protein	Regulation	Mean score
Xirp2	497771	xin actin-binding repeat containing 2	Up	3.97
ANP	24602	atrial natriuretic peptide	Up	3.95
CSRP2	29317	cysteine and glycine-rich protein 2	Up	2.77
PTGIS	25527	prostaglandin I2 (prostacyclin) synthase	Up	2.66
C11Orf17	361624	A kinase (PRKA) interacting protein 1	Up	2.48
FHL1	25177	four and a half LIM domains 1	Up	2.44
TIMP1	116510	tissue inhibitor of metalloproteinase 1	Up	2.42
TNFRSF12a	302965	tumor necrosis factor receptor superfamily, member 12A	Up	2.13
AVPI1	171386	arginine vasopressin-induced 1	Up	2.11
LGALS3	83781	lectin, galactoside-binding, soluble, 3	Up	1.99
ENAH	360891	enabled homolog (Drosophila)	Up	1.98
Dhrs7c	287411	dehydrogenase/reductase (SDR family) member 7C	Down	-6.21
CXCL11	305236	chemokine (C-X-C motif) ligand 11	Down	-2.92
RXRG	83574	retinoid X receptor gamma	Down	-2.37
SLC4a1ap	298805	solute carrier family 4 (anion exchanger), member 1, adaptor protein	Down	-2.06
SLC2a4	25139	solute carrier family 2 (facilitated glucose transporter), member 4	Down	-1.95
LRP16	246233	MACRO domain containing 1	Down	-1.71
PINK1	298575	PTEN induced putative kinase 1	Down	-1.68
CABC1	360887	chaperone, ABC1 activity of bc1 complex like (S. pombe)	Down	-1.66
HADHSC	113965	hydroxyacyl-CoA dehydrogenase	Down	-1.63
ECH1	64526	enoyl CoA hydratase 1, peroxisomal	Down	-1.60

The mean score is the average fold up- or down regulation.

obtained. The *in vivo* specific genes were enriched for extracellular matrix ($p=4.4x10^{-7}$), wounding ($p=2.0x10^{-6}$) and stress ($p=2.6x10^{-5}$) (Table 5). This included the highly expressed genes LTBP, POSTN, fibronectin (FN1) and LOXL1. In contrast the *in vitro* specific genes were enriched for genes involved in terpenoid backbone ($p=8.9x10^{-11}$) and steroid biosynthesis ($p=1.4x10^{-6}$) pathways (Table 5). Especially in PE and ET-1 treated cells nearly all genes of these pathways, which together are involved in sterol, cholesterol and steroid biosynthesis, were upregulated (see Supplemental figure S1).

Table 4. *In vitro* and *in vivo* specific genes

In vitro only			In vivo only		
Gene	**Entrez Id**	**Regulation**	**Gene**	**Entrez Id**	**Regulation**
PNOC	25516	Up	LTBP2	59106	Up
SCN3b	245956	Up	POSTN	361945	Up
INHA	24504	Up	NUPR1	113900	Up
ACTC1	29275	Up	FN1	25661	Up
RGD1560242	499334	Up	SERPINE2	29366	Up
RND1	362993	Up	LOXL1	315714	Up
MYBPC2	292879	Up	MGC72614	310540	Up
IFIT1	294090	Up	MFAP5	362429	Up
YIP1B	364147	Up	CTGF	64032	Up
SLPI	84386	Up	ANXA2	56611	Up
ANGPT2	89805	Down	TXN2	79462	Down
PTPRR	94202	Down	ACAT1	25014	Down
ASB11	302666	Down	PFKM	65152	Down
LOC191574	191574	Down	ALDH5a1	291133	Down
Adhfe1	362474	Down	LDHD	307858	Down
CXCL13	498335	Down	ISOC1	364879	Down
RGD1562717	363767	Down	NDE1	83836	Down
SMOC2	292401	Down	SESn1	294518	Down
CITED1	64466	Down	GOT2	25721	Down
PTN	24942	Down	RGD1565358	314759	Down

Top ten genes with highest up- or downregulation specific for at least two *in vitro* conditions (left half) or specific for the *in vivo* conditions only (right half). All listed genes with (1.5 fold, $p<0.05$).

Discussion

Cardiomyocyte growth (hypertrophy) is one of the hallmarks of HF development [12, 13]. Here we investigated gene expression profiles of *in vitro* cultured cardiomyocytes treated with hypertrophy inducing agents (PE, ET-1 and Iso) and *in vivo* HF models (hypertensive Ren2 rats and post-MI rats). This approach yielded large data sets of differentially expressed genes. By overlapping them a concise set of genes, including numerous known HF markers, were obtained. Interestingly, this set also included a number of interesting genes not linked to hypertrophy/HF before. Analysis of differential gene expression in cardiomyocytes stimulated with hypertrophy inducing agents and in two rat HF models generated large lists of differentially expressed genes. By combining these expression data sets we obtained a concise list of 62 differentially expressed genes. This list included most known HF markers, including ANP, BNP, TIMP1, FHL1, LGALS3 (Table 3 and Supplementary gene list 3), indicating that this is a powerful approach to identify differentially expressed genes that are specifically linked to hypertrophy and HF development. This list also comprised genes that have recently been linked to cardiac remodeling, like TNFRSF12a (also termed Fn14) [4], indicating that this approach could uncover new HF genes. In fact this list contained a number of genes regulated in a similar fashion that have previously not been linked to hypertrophy and HF development. This list includes PTGIS, a gene for which myocardial infarction, hypertension and stroke associated polymorphisms have been identified by GWAS

Table 5. GGO analysis of *in vitro* and *in vivo* specific genes

Specificity	Category	P value	Genes
In vivo	Extracellular matrix	$4.4x10^{-7}$	Ltbp2, FN1, Loxl1, Col3a1, Serpine2, Mgp, Ctgf, Anxa2
	Wounding	$2.0x10^{-6}$	FN1, Nupr1, Col3a1, Serpine2, Serping1, Anxa2, Anxa1, Aif1, C1s
	Stress response	$2.6x10^{-5}$	FN1, Nupr1, Col3a1, Serpine2, Serping1, Ctgf, Anxa2, Anxa1, Aif1, GPX3, C1s, Prnp
In vitro	Cholesterol biosynthesis and metabolism	$7.8x10^{-12}$	Dhcr24, Idi1, Hmgcs1, Cyp51, Dhcr7, Fdps, Fdft1, Pmvk, Mvd, Cln8
	Sterol biosynthesis and metabolism	$3.0x10^{-11}$	Dhcr24, Idi1, Hmgcs1, Cyp51, Dhcr7, Fdps, Fdft1, Pmvk, Mvd, Cln8
	Steroid biosynthesis and metabolism	$4.0x10^{-8}$	Dhcr24, Idi1, Hmgcs1, Cyp51, Dhcr7, Fdps, Fdft1, Pmvk, Mvd, Cln8, Por

studies [21], AKIP1, an adaptor protein in the PKA-NFkB pathways [11, 27] and the short chain dehydrogenase/reductase Dhrs7c (downregulated). Our inventory could therefore be an important starting point for the investigation of novel genes associated with HF.

Our investigations also revealed clear differences between *in vitro* and *in vivo* experiments. GGO analysis showed a strong enrichment for extracellular matrix, wounding and stress specific genes in the *in vivo* list. This included the highly expressed genes LTBP, POSTN, FN1 and LOXL1. These genes are predominantly expressed by fibroblasts or other non-cardiomyocytes in the heart and explains the absence in the *in vitro* cardiomyocyte experiments. This list is therefore of particular interest since it contains many differentially expressed HF genes that are specific for non-cardiomyocytes. Further analysis of uncharacterized genes present in this list, like MGC72614 (FAM198B), could therefore be a rewarding opportunity. GGO analysis also revealed a strong enrichment of genes involved in the terpenoid and steroid biosynthesis pathways that are specifically upregulated *in vitro* after hypertrophic stimulation. Since these pathways are essential for the novo cholesterol synthesis, it might suggest that there is an increased requirement for cholesterol during hypertrophic growth of cardiomyocytes.

Also marked differences were observed within the *in vivo* models. Carbonic anhydrase III (CA3) for example was almost 50 fold induced in the post-MI group, but not in the Ren2 group. CA3 is the major cytosolic carbonic anhydrase in muscle and liver tissue [28]. It probably functions to protect proteins from oxidation catalyzed by iron-containing degradation products of hemoglobin and myoglobin and/or regulating intracellular pH [28, 25]. Although not essential under normal conditions, it might become important in certain diseased states [15]. It could be envisioned that acidification and iron containing degradation products might be a major problem in post-MI hearts, explaining the strong upregulation in one (post-MI), but not the other (Ren2) model. The absence of induction in the Ren2 model indicates that fibrotic processes in post-MI and Ren2 animals are not similar. In the Ren2 group suprabasin (RGD1562305) expression was specifically increased. This extracellular protein was originally suggested to play a role in striated epithelia [20], but was recently also shown to be strongly induced in muscle of exercised dogs [2]. Together with our observation, this may suggest that suprabasin also plays a role in heart and muscle function under stress related conditions. Whether the absence in the post-MI HF group suggests etiologic specificity is not known, but it might be rewarding to investigate plasma levels of this secreted protein in different HF groups.

Several features of the experiments are important in the interpretation of the present data. First we used neonatal rat cardiomyocytes for *in vitro* hypertrophy and compared these data with *in vivo* adult rat HF models. Therefore some of the differences between the *in vitro*

and *in vivo* data could be related to the age of the cells, neonatal versus adult. We made this choice because neonatal rat cardiomyocytes are used as an established model for *in vitro* cardiomyocyte hypertrophy. Some differences could be related to the timing of the experiments. *In vitro* hypertrophy induction only lasted 24 hours and therefore some of the changes might be related to early responses in contrast to the *in vivo* experiments. As pointed out for KCNE1, some of the effects might also be temporal or related to the severity of HF and hence be present in one, but not the other group. As with most screens we also missed some known HF markers, like SERCA, indicating that although comprehensive the generated lists are not complete. Except for ANP we did not confirm the microarray data by direct, specific quantitation of mRNAs or proteins in the heart or cardiomyocytes.

In conclusion, we have identified a comprehensive set of genes that are differentially expressed in *in vitro* cardiomyocyte models for hypertrophy and in animal HF models. This list includes a large number of genes known to be associated with HF, as well as potential new targets, like AKIP1, for future analysis. Furthermore, we have identified a large number of genes that appear to be specific for particular conditions. This stresses again that we have to be careful with extrapolations from one to another model or condition. These datasets should prove invaluable for a further analysis of cardiac diseases in particular pathological hypertrophy and HF development.

Acknowledgements

We like to thank Bahram Sanjabi, who performed the gene array hybridization and detection at the Department of Genetics, University Medical Center Groningen, The Netherlands.

References

1. Asakura M and Kitakaze M. Global gene expression profiling in the failing myocardium. *Circ.J.* 73: 9: 1568-1576, 2009.

2. Brass EP, Peters MA, Hinchcliff KW, He YD and Ulrich RG. Temporal pattern of skeletal muscle gene expression following endurance exercise in Alaskan sled dogs. *J.Appl.Physiol.* 107: 2: 605-612, 2009.

3. Cheng TH, Chen JJ, Shih NL, Lin JW, Liu JC, Chen YL, Chen CH and Chen JJ. Mechanical stretch induces endothelial nitric oxide synthase gene expression in neonatal rat cardiomyocytes. *Clin.Exp.Pharmacol.Physiol.* 36: 5-6: 559-566, 2009.

4. Chorianopoulos E, Heger T, Lutz M, Frank D, Bea F, Katus HA and Frey N. FGF-inducible 14-kDa protein (Fn14) is regulated via the RhoA/ROCK kinase pathway in cardiomyocytes and mediates nuclear factor-kappaB activation by TWEAK. *Basic Res.Cardiol.* 105: 2: 301-313, 2010.

5. Cullingford TE, Markou T, Fuller SJ, Giraldo A, Pikkarainen S, Zoumpoulidou G, Alsafi A, Ekere C, Kemp TJ, Dennis JL, Game L, Sugden PH and Clerk A. Temporal regulation of expression of immediate early and second phase transcripts by endothelin-1 in cardiomyocytes. *Genome Biol.* 9: 2: R32, 2008.

6. de Boer RA, Lok DJ, Jaarsma T, van der Meer P, Voors AA, Hillege HL and van Veldhuisen DJ. Predictive value of plasma galectin-3 levels in heart failure with reduced and preserved ejection fraction. *Ann.Med.* 43: 1: 60-68, 2011.

7. de Boer RA, Pokharel S, Flesch M, van Kampen DA, Suurmeijer AJ, Boomsma F, van Gilst WH, van Veldhuisen DJ and Pinto YM. Extracellular signal regulated kinase and SMAD signaling both mediate the angiotensin II driven progression towards overt heart failure in homozygous TGR(mRen2)27. *J.Mol.Med.(Berl)* 82: 10: 678-687, 2004.

8. de Boer RA, Voors AA, Muntendam P, van Gilst WH and van Veldhuisen DJ. Galectin-3: a novel mediator of heart failure development and progression. *Eur.J.Heart Fail.* 11: 9: 811-817, 2009.

9. Feng Y, Vom Hagen F, Wang Y, Beck S, Schreiter K, Pfister F, Hoffmann S, Wagner P, Seeliger M, Molema G, Deutsch U and Hammes HP. The absence of angiopoietin-2 leads to abnormal vascular maturation and persistent proliferative retinopathy. *Thromb.Haemost.* 102: 1: 120-130, 2009.

10. Frank D, Kuhn C, Brors B, Hanselmann C, Ludde M, Katus HA and Frey N. Gene expression pattern in biomechanically stretched cardiomyocytes: evidence for a stretch-specific gene program. *Hypertension* 51: 2: 309-318, 2008.

11. Gao N, Asamitsu K, Hibi Y, Ueno T and Okamoto T. AKIP1 enhances NF-kappaB-dependent gene expression by promoting the nuclear retention and phosphorylation of p65. *J.Biol.Chem.* 283: 12: 7834-7843, 2008.

12. Heineke J and Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat.Rev.Mol.Cell Biol.* 7: 8: 589-600, 2006.

13. Hill JA and Olson EN. Cardiac plasticity. *N.Engl.J.Med.* 358: 13: 1370-1380, 2008.

14. Iwaki K, Sukhatme VP, Shubeita HE and Chien KR. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. *J.Biol.Chem.* 265: 23: 13809-13817, 1990.

15. Kim G, Lee TH, Wetzel P, Geers C, Robinson MA, Myers TG, Owens JW, Wehr NB, Eckhaus MW, Gros G, Wynshaw-Boris A and Levine RL. Carbonic anhydrase III is not required in the mouse for normal growth, development, and life span. *Mol.Cell.Biol.* 24: 22: 9942-9947, 2004.

16. Kjaer A and Hesse B. Heart failure and neuroendocrine activation: diagnostic, prognostic and therapeutic perspectives. *Clin.Physiol.* 21: 6: 661-672, 2001.

17. Lee MA, Bohm M, Paul M, Bader M, Ganten U and Ganten D. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. *Am.J.Physiol.* 270: 6 Pt 1: E919-29, 1996.

18. Lipsic E, Westenbrink BD, van der Meer P, van der Harst P, Voors AA, van Veldhuisen DJ, Schoemaker RG and van Gilst WH. Low-dose erythropoietin improves cardiac function in experimental heart failure without increasing haematocrit. *Eur.J.Heart Fail.* 10: 1: 22-29, 2008.

19. Lu B, Mahmud H, Maass AH, Yu B, van Gilst WH, de Boer RA and Sillje HH. The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy in vitro. *PLoS One* 5: 9: e12963, 2010.

20. Matsui T, Hayashi-Kisumi F, Kinoshita Y, Katahira S, Morita K, Miyachi Y, Ono Y, Imai T, Tanigawa Y, Komiya T and Tsukita S. Identification of novel keratinocyte-secreted peptides dermokine-alpha/-beta and a new stratified epithelium-secreted protein gene complex on human chromosome 19q13.1. *Genomics* 84: 2: 384-397, 2004.

21. Nakayama T. Genetic polymorphisms of prostacyclin synthase gene and cardiovascular disease. *Int.Angiol.* 29: 2 Suppl: 33-42, 2010.

22. Norris RA, Borg TK, Butcher JT, Baudino TA, Banerjee I and Markwald RR. Neonatal and adult cardiovascular pathophysiological remodeling and repair: developmental role of periostin. *Ann.N.Y.Acad.Sci.* 1123: 30-40, 2008.

23. Oeseburg H, de Boer RA, Buikema H, van der Harst P, van Gilst WH and Sillje HH. Glucagon-like peptide 1 prevents reactive oxygen species-induced endothelial cell senescence through the activation of protein kinase A. *Arterioscler.Thromb.Vasc.Biol.* 30: 7: 1407-1414, 2010.

24. Rajabi M, Kassiotis C, Razeghi P and Taegtmeyer H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail.Rev.* 12: 3-4: 331-343, 2007.

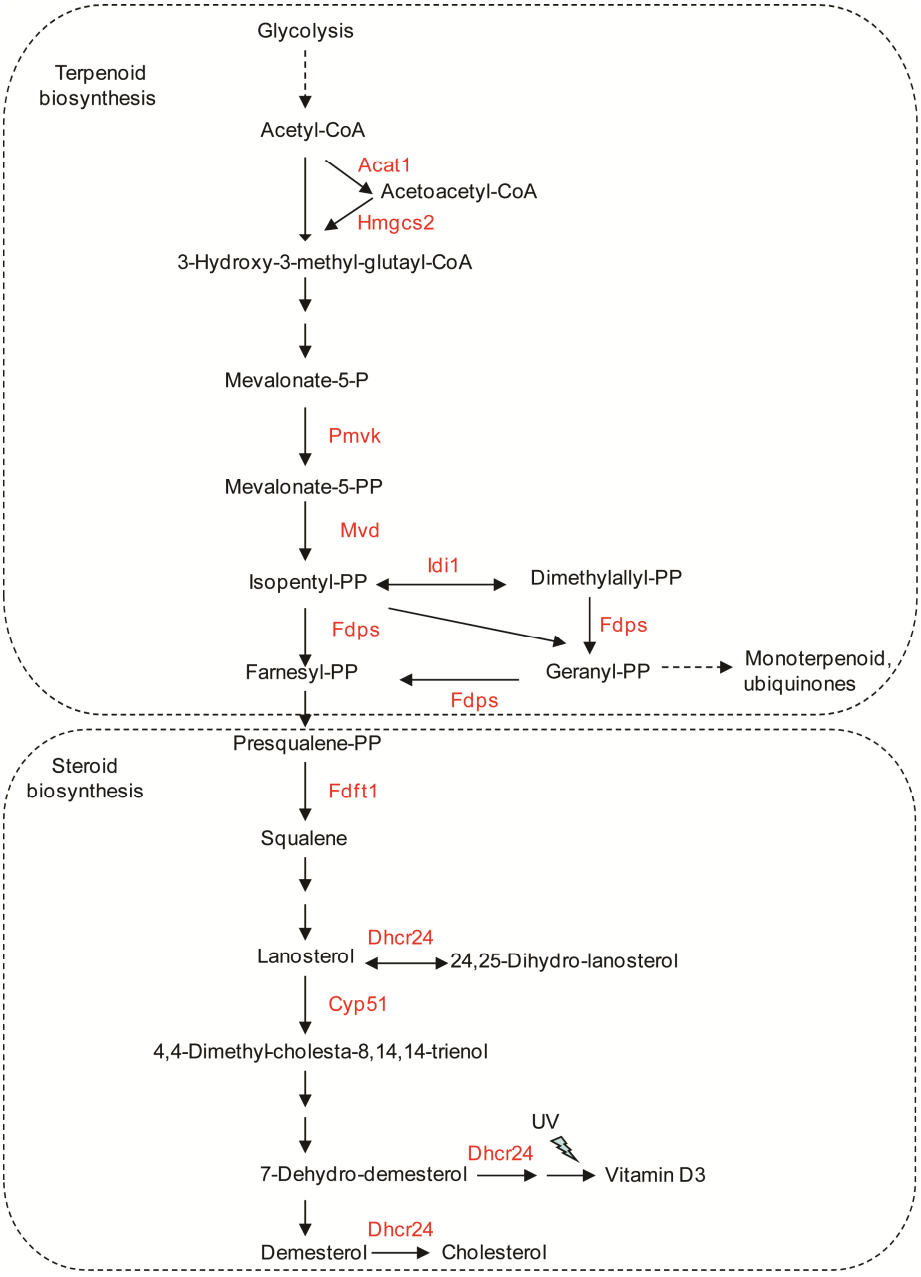
25. Roy P, Reavey E, Rayne M, Roy S, Abed El Baky M, Ishii Y and Bartholomew C. Enhanced sensitivity to hydrogen peroxide-induced apoptosis in Evi1 transformed Rat1 fibroblasts due to repression of carbonic anhydrase III. *FEBS J.* 277: 2: 441-452, 2010.

26. Ruifrok WP, Qian C, Sillje HH, van Goor H, van Veldhuisen DJ, van Gilst WH and de Boer RA. Heart failure-associated anemia: bone marrow dysfunction and response to erythropoietin. *J.Mol.Med.(Berl)* 89: 4: 377-387, 2011.

27. Sastri M, Barraclough DM, Carmichael PT and Taylor SS. A-kinase-interacting protein localizes protein kinase A in the nucleus. *Proc.Natl.Acad.Sci.U.S.A.* 102: 2: 349-354, 2005.

28. Wistrand PJ. Carbonic anhydrase III in liver and muscle of male rats purification and properties. *Ups.J.Med.Sci.* 107: 2: 77-88, 2002.

Supplemental data



SFigure 1. Terpenoid and steroid biosynthesis pathways

Differentially expressed genes identified in our *in vitro* models are marked in grey.

Gene list S1. Differentially expressed genes identified in *in vivo* post-MI HF and Ren2 rat models (fold change >1.5 and p<0.05)

Gene	Genbank ID	Protein	Regulation	MI	Ren2
Ltbp2	59106	latent transforming growth factor beta binding protein 2	up	24.93	8.64
Postn	361945	periostin, osteoblast specific factor	up	13.50	8.82
Nppa	24602	natriuretic peptide precursor type A	up	5.96	9.39
Pap	24618	pancreatitis-associated protein	up	4.59	5.91
Nupr1	113900	nuclear protein 1	up	5.09	5.06
Col8a1	304021	procollagen, type VIII, alpha 1	up	6.99	3.14
Fn1	25661	fibronectin 1	up	5.69	2.60
Serpine2	29366	serine (or cysteine) proteinase inhibitor, clade E, member 2	up	3.35	4.45
Timp1	116510	tissue inhibitor of metallopeptidase 1	up	4.50	2.78
RGD1306959	361624	similar to C11orf17 protein	up	2.58	4.42
Loxl1	315714	lysyl oxidase-like 1	up	4.51	2.33
Col3a1	84032	procollagen, type III, alpha 1	up	3.35	3.26
Pi16	294312	protease inhibitor 16	up	3.50	2.67
MGC72614	310540	hypothetical LOC310540	up	2.55	3.17
Mfap5	362429	microfibrillar associated protein 5	up	3.94	1.71
Ifitm1	293618	interferon induced transmembrane protein 1	up	2.73	2.79
Maoa	29253	monoamine oxidase A	up	2.33	2.91
Lgals3	83781	lectin, galactose binding, soluble 3	up	2.99	2.08
Ctgf	64032	connective tissue growth factor	up	2.83	2.11
Anxa2	56611	annexin A2	up	2.83	1.86
Prss23	308807	protease, serine, 23	up	2.51	2.07
Serping1	295703	serine (or cysteine) peptidase inhibitor, clade G, member 1	up	2.92	1.64
Mgp	25333	matrix Gla protein	up	2.96	1.60
Aif1	29427	allograft inflammatory factor 1	up	2.39	1.94
Col14a1	314981	procollagen, type XIV, alpha 1	up	2.40	1.86
Anxa1	25380	annexin A1	up	2.41	1.62
Gpx3	64317	glutathione peroxidase 3	up	2.23	1.75
Ecm1	116662	extracellular matrix protein 1	up	2.05	1.90
Wbp5	294067	WW domain binding protein 5	up	1.78	2.13
Cd248	293669	CD248 antigen, endosialin	up	2.22	1.55
Fuca	24375	fucosidase, alpha-L- 1, tissue	up	1.56	2.19

Gene	Genbank ID	Protein	Regulation	MI	Ren2
Scpep1	114861	serine carboxypeptidase 1	up	2.07	1.56
Sulf2	311642	sulfatase 2	up	2.02	1.61
C1s	192262	complement component 1, s subcomponent	up	1.96	1.52
Grn	29143	granulin	up	1.72	1.66
Vim	81818	vimentin	up	1.85	1.52
Ccnd1	58919	cyclin D1	up	1.70	1.60
Aldh1a1	24188	aldehyde dehydrogenase family 1, member A1	up	1.53	1.69
Ankrd1	27064	ankyrin repeat domain 1	up	1.55	1.67
Prnp	24686	prion protein	up	1.60	1.61
Txn2	79462	thioredoxin 2	down	-1.76	-3.23
Aqp7	29171	aquaporin 7	down	-2.77	-1.57
Boll	302920	bol, boule-like	down	-1.98	-2.24
Slc2a4	25139	solute carrier family 2 (facilitated glucose transporter), member 4	down	-2.11	-2.07
Acat1	25014	acetyl-coenzyme A acetyltransferase 1	down	-1.85	-2.32
Lrp16	246233	LRP16 protein	down	-2.20	-1.77
Pfkm	65152	phosphofructokinase, muscle	down	-2.13	-1.62
Aldh5a1	291133	aldehyde dehydrogenase family 5, subfamily A1, transcript variant 3	down	-1.68	-2.06
Hrc	292905	histidine rich calcium binding protein	down	-1.95	-1.68
Ldhd	307858	lactate dehydrogenase D	down	-2.06	-1.53
Rxrg	83574	retinoid X receptor gamma	down	-1.96	-1.59
Isoc1	364879	isochorismatase domain containing 1	down	-1.51	-1.89
Nde1	83836	nuclear distribution gene E homolog 1	down	-1.51	-1.88
Mlycd	85239	malonyl-CoA decarboxylase	down	-1.85	-1.53
Sesn1	294518	sestrin 1	down	-1.54	-1.62
Got2	25721	glutamate oxaloacetate transaminase 2, mitochondrial	down	-1.55	-1.52
RGD1565358	314759	similar to genes associated with retinoid-IFN-induced mortality 19	down	-1.52	-1.52
Polr2i	292778	polymerase (RNA) II (DNA directed) polypeptide I	down/up	-1.52	1.52

Gene list S2. Differentially expressed genes identified in *in vitro* cardiomyocyte cultures treated with PE, Iso or ET-1 (fold change >1.5 and p<0.05)

Gene	Genebank ID	Protein	Regulation	PE	ET-1	Iso
Wfdc1	171112	WAP four-disulfide core domain 1	up	11.05	3.89	5.30
Scn3b	245956	sodium channel, voltage-gated, type III, beta	up	6.82	4.37	4.98
LOC497771	497771	hypothetical gene supported by NM_201989	up	5.85	4.14	3.29
Inha	24504	inhibin alpha	up	5.62	2.13	4.48
Actc1	29275	actin alpha cardiac 1	up	5.05	3.37	3.37
RGD1560242	499334	similar to RIKEN cDNA 1700028P14	up	4.84	1.83	3.69
Rnd1	362993	Rho family GTPase 1	up	4.25	2.06	3.91
Ptgis	25527	prostaglandin I2 (prostacyclin) synthase	up	4.10	2.28	2.77
Fhl1	25177	four and a half LIM domains 1, transcript variant 2, mRNA.	up	3.57	2.43	2.29
Mybpc2	292879	myosin binding protein C, fast-type	up	3.47	2.11	3.41
Igsf9	304982	immunoglobulin superfamily, member 9	up	3.39	1.72	2.66
RGD1310766	364147	similar to YIP1B	up	3.19	2.09	2.96
Hbegf	25433	heparin-binding EGF-like growth factor	up	3.18	1.97	2.26
Tnfrsf12a	302965	tumor necrosis factor receptor superfamily, member 12a	up	2.99	1.86	2.21
Irf6	364081	interferon regulatory factor 6	up	2.86	1.69	2.39
Gadd45g	291005	growth arrest and DNA-damage-inducible 45 gamma	up	2.70	1.92	2.38
Edg7	360355	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 7	up	2.67	1.74	2.73
Cspg4	81651	chondroitin sulfate proteoglycan 4	up	2.59	1.94	1.70
Lamc2	192362	lamimin, gamma 2	up	2.55	1.85	1.73
Acta1	29437	actin, alpha 1, skeletal muscle	up	2.50	2.06	1.99
Gloxd1	313521	glyoxalase domain containing 1	up	2.49	2.26	2.48
G7c	309611	G7c protein	up	2.49	1.67	3.27
Cyp51	25427	cytochrome P450, subfamily 51	up	2.48	1.89	1.55
Avpi1	171386	arginine vasopressin-induced 1	up	2.47	1.96	1.98
Hspb3	78951	heat shock 27kDa protein 3	up	2.44	2.01	2.20
Olfm1	93667	olfactomedin 1	up	2.37	1.58	1.66
Adprhl1	290880	ADP-ribosylhydrolase like 1	up	2.36	1.84	1.96
Slc16a3	80878	solute carrier family 16 (monocarboxylic acid transporters), member 3	up	2.34	1.82	1.79

Gene	Genebank ID	Protein	Regulation	PE	ET-1	Iso
Enah	360891	enabled homolog (Drosophila)	up	2.30	1.62	1.93
Idi1	89784	isopentenyl-diphosphate delta isomerase	up	2.30	1.90	1.66
Pdlim5	64353	PDZ and LIM domain 5	up	2.27	1.66	1.50
Lrrc8c	289443	leucine rich repeat containing 8 family, member C	up	2.17	1.54	1.77
Csrp3	117505	cysteine and glycine-rich protein 3	up	2.12	1.70	1.96
Dnajb5	313811	DnaJ (Hsp40) homolog, subfamily B, member 5	up	2.07	1.52	1.97
LOC365025	365025	similar to alpha tubulin subunit	up	2.03	1.64	1.71
Dhcr24	298298	24-dehydrocholesterol reductase	up	2.03	2.04	1.89
RGD1306959	361624	similar to C11orf17 protein	up	2.03	1.64	1.71
Itgb1bp2	317258	integrin beta 1 binding protein 2	up	2.03	1.76	1.81
Tuba4a	316531	tubulin, alpha 4A	up	2.02	1.75	1.86
RGD1311552	311355	similar to KIAA0377-like protein , transcript variant 4	up	1.98	1.53	1.66
Pib5pa	171088	phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	up	1.96	1.74	2.04
Dmn	308709	desmuslin, transcript variant 2	up	1.95	1.70	1.82
Mgst2	295037	microsomal glutathione S-transferase 2	up	1.95	1.72	1.67
Por	29441	P450 (cytochrome) oxidoreductase	up	1.84	1.53	1.59
Abra	286965	actin-binding Rho activating protein	up	1.84	1.55	1.54
Gnao	50664	guanine nucleotide binding protein, alpha o	up	1.70	1.58	1.70
Angpt2	89805	angiopoietin 2	down	-4.66	-1.92	-4.01
LOC191574	191574	3-alpha-hydroxysteroid dehydrogenase	down	-3.18	-2.45	-2.19
RGD1562717	363767	similar to ABI gene family, member 3 (NESH) binding protein	down	-2.87	-2.46	-1.67
Smoc2	292401	SPARC related modular calcium binding 2 , transcript variant 2	down	-2.75	-1.89	-2.15
RGD1311203	305472	similar to HGFL protein	down	-2.75	-1.60	-1.65
Cdkn1c	246060	cyclin-dependent kinase inhibitor 1C (P57) , transcript variant 1, mRNA.	down	-2.56	-1.56	-1.65
Irf7	293624	interferon regulatory factor 7	down	-2.48	-1.78	-1.76
Agtr1a	24180	angiotensin II receptor, type 1 (AT1A)	down	-2.44	-1.59	-1.84
Prp2	293634	proline-rich protein PRP2	down	-2.41	-2.09	-1.71
CXCL13	498335	similar to Small inducible cytokine B13 precursor	down	-2.40	-2.85	-1.99
Eln	25043	elastin	down	-2.11	-1.64	-1.74
RGD1560587	316539	similar to Eph receptor A4	down	-2.00	-1.65	-1.80
Zc3h6	311415	zinc finger CCCH type containing 6	down	-1.93	-1.66	-1.98

Gene	Genebank ID	Protein	Regulation	PE	ET-1	Iso
Kcnk3	29553	potassium channel, subfamily K, member 3	down	-1.87	-1.73	-1.97
Gpm6a	306439	glycoprotein m6a	down	-1.86	-1.62	-1.58
Ech1	64526	enoyl coenzyme A hydratase 1, peroxisomal	down	-1.86	-1.59	-1.60
Fmod	64507	fibromodulin	down	-1.84	-1.56	-1.55
Hamp	84604	hepcidin antimicrobial peptide	down	-1.79	-1.65	-1.69
RGD1559432	498937	RGD1559432	down	-1.76	-1.64	-1.65
Mllt3	114510	myeloid/lymphoid or mixed-lineage leukemia,translocated to, 3	down	-1.75	-1.53	-1.56
Ptgds	25526	prostaglandin D2 synthase	down	-1.73	-1.54	-1.53
Npy	24604	neuropeptide Y	down	-1.68	-2.34	-2.10
Plagl1	25157	pleiomorphic adenoma gene-like 1	down	-1.54	-1.72	-1.58
Okl38	171493	pregnancy-induced growth inhibitor	down/up	-2.00	-1.56	2.69

Gene list S3. Comparison of the *in vivo* and *in vitro* gene lists (Gene list S1 versus Gene list S2)

Gene	Genebank ID	Protein	*In vitro*						*In vivo*			
			PE		ET-1		Iso		MI		Ren2	
			Regulation	Fold	Regulation	Fold	Regulation	Fold	Regulation	Fold	Regulation	Fold
LOC497771	497771	hypothetical gene supported by NM_201989	up	5.85	up	4.14	up	3.29	up	2.81	up	3.78
Nppa	24602	natriuretic peptide precursor type A	up	1.55	up	1.52	up	1.35	up	5.96	up	9.39
Csrp2	29317	cysteine and glycine-rich protein 2	up	1.55	up	1.14	up	2.66	up	6.56	up	1.94
Ptgis	25527	prostaglandin I2 (prostacyclin) synthase	up	4.10	up	2.28	up	2.77	up	2.50	up	1.67
RGD1306959	361624	similar to C11orf17 protein	up	2.03	up	1.64	up	1.71	up	2.58	up	4.42
Fhl1	25177	four and a half LIM domains 1 , transcript variant 2	up	3.57	up	2.43	up	2.29	up	2.50	up	1.40
Timp1	116510	tissue inhibitor of metallopeptidase 1	up	1.78	up	1.57	up	1.48	up	4.50	up	2.78
Tnfrsf12a	302965	tumor necrosis factor receptor superfamily, member 12a	up	2.99	up	1.86	up	2.21	up	1.74	up	1.87
Avpi1	171386	arginine vasopressin-induced 1	up	2.47	up	1.96	up	1.98	up	2.05	up	2.09
Lgals3	83781	lectin, galactose binding, soluble 3	up	1.87	up	1.45	up	1.55	up	2.99	up	2.08
Enah	360891	enabled homolog (Drosophila) (Enah)	up	2.30	up	1.62	up	1.93	up	1.45	up	2.60
Bok	29884	Bcl-2-related ovarian killer protein	up	2.31	up	1.59	up	1.95	up	2.38	up	1.66
Hspb3	78951	heat shock 27kDa protein 3	up	2.44	up	2.01	up	2.20	up	1.19	up	1.64
Gnao	50664	guanine nucleotide binding protein, alpha o	up	1.70	up	1.58	up	1.70	up	2.58	up	1.84
Ecm1	116662	extracellular matrix protein 1	up	2.13	up	1.34	up	1.90	up	2.05	up	1.90
Argbp2	114901	Arg/Abl-interacting protein ArgBP2	up	2.05	up	1.44	up	1.70	up	1.50	up	1.98

Gene	Genebank ID	Protein	*In vitro*						*In vivo*			
			PE		ET-1		Iso		MI		Ren2	
			Regulation	Fold	Regulation	Fold	Regulation	Fold	Regulation	Fold	Regulation	Fold
Nppb	25105	natriuretic peptide precursor type B	up	1.44	up	1.55	up	1.57	up	1.42	up	2.20
Sod3	25352	superoxide dismutase 3, extracellular	up	1.94	up	1.02	up	2.01	up	1.91	up	1.04
Flnc	362332	filamin C, gamma (actin binding protein 280)	up	1.77	up	1.38	up	1.68	up	1.16	up	1.60
LOC499702	499702	similar to Synaptopodin-2	up	1.55	up	1.50	up	1.22	up	1.26	up	1.67
Dhrs7c	287411	dehydrogenase/reductase (SDR family) member 7C	down	-4.12	down	-1.02	down	-20.18	down	-3.50	down	-2.22
Cxcl11	305236	chemokine (C-X-C motif) ligand 11	down	-3.17	down	-1.35	down	-5.56	down	-2.87	down	-1.63
Rxrg	83574	retinoid X receptor gamma	down	-3.12	down	-1.69	down	-3.47	down	-1.96	down	-1.59
Slc4a1ap	298805	solute carrier family 4 (anion exchanger), member 1, adaptor protein	down	-2.82	down	-1.30	down	-3.24	down	-1.78	down	-1.17
Slc2a4	25139	solute carrier family 2 (facilitated glucose transporter), member 4	down	-1.85	down	-1.17	down	-2.56	down	-2.11	down	-2.07
Lrp16	246233	LRP16 protein	down	-1.65	down	-1.52	down	-1.40	down	-2.20	down	-1.77
Pink1	298575	PTEN induced putative kinase 1	down	-2.19	down	-1.28	down	-1.84	down	-1.79	down	-1.30
Cabc1	360887	chaperone, ABC1 activity of bc1 complex like	down	-1.82	down	-1.29	down	-1.75	down	-2.03	down	-1.40
Hadhsc	113965	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	down	-1.73	down	-1.20	down	-1.55	down	-2.15	down	-1.52
Ech1	64526	enoyl coenzyme A hydratase 1, peroxisomal	down	-1.86	down	-1.59	down	-1.60	down	-1.70	down	-1.24
Adra1b	24173	adrenergic receptor, alpha 1b	down	-1.68	down	-1.65	down	-1.03	down	-1.67	down	-1.63
Nudt4	94267	nudix (nucleoside diphosphate linked moiety X)-type motif 4	down	-1.59	down	-1.40	down	-1.53	down	-1.40	down	-1.71

Gene	Genebank ID	Protein	*In vitro*						*In vivo*			
			PE		ET-1		Iso		MI		Ren2	
			Regulation	Fold	Regulation	Fold	Regulation	Fold	Regulation	Fold	Regulation	Fold
LOC306805	306805	similar to asporin precursor	down	-1.71	down	-1.76	down	-1.23	up	2.61	up	1.25
Col14a1	314981	procollagen, type XIV, alpha 1	down	-1.28	down	-1.12	down	-1.50	up	2.40	up	1.86
Dap	64322	death-associated protein	down	-1.52	down	-1.39	down	-1.33	up	1.88	up	1.27
Wfdc1	171112	WAP four-disulfide core domain 1	up	11.05	up	3.89	up	5.30	down	-1.12	down	-2.51
Eno3	25438	enolase 3, beta	up	2.27	up	1.09	up	1.55	down	-1.85	down	-1.13
Pdlim3	114108	PDZ and LIM domain 3	up	1.98	up	1.68	up	1.46	down	-1.01	down	-1.50
Itga7	81008	integrin alpha 7	up	1.62	up	1.67	down	-1.12	down	-1.50	down	-1.07
Aga	290923	aspartylglucosaminidase	down	-1.59	down	-1.28	down	-1.45	up	1.62	up	1.10

Chapter 5

DHRS7C, a novel cardiomyocyte expressed gene that is downregulated by adrenergic stimulation and in heart failure

Bo Lu
Wardit Tigchelaar
Willem P.T. Ruifrok
Wiek H. van Gilst
Rudolf A. de Boer
Herman H.W. Silljé

Eur J Heart Fail. 2012 Jan;14(1):5-13.

Abstract

Aims Although cardiac diseases account for the highest mortality and morbidity rates in Western society, there is still a considerable lack in our knowledge of genes that contribute to cardiac (dys)function. Here we screened for gene expression profiles correlated to heart failure. **Methods and results** By expression profiling we identified a novel gene, termed *DHRS7c*, which was significantly downregulated by adrenergic stimulation and in heart failure models. Dhrs7c is a short chain dehydrogenase/reductase (SDR) and localized to the endo/sarcoplasmatic reticulum. Dhrs7c is strongly conserved in vertebrates, and mRNA and protein expression levels were highest in heart and skeletal muscle followed by skin, but was not detectable in other organs. *In vitro,* both α- and β-adrenergic stimulation repressed Dhrs7c expression in neonatal cardiomyocytes and this could be mimicked by the direct activation of PKC and adenylate cyclase, the respective intracellular targets of these hormones. In contrast, endothelin-1, which also provoked strong hypertrophy development *in vitro*, did not repress Dhrs7c expression. The latter suggests adrenergic specificity and indicates that downregulation is not a prerequisite for hypertrophy development. Also *in vivo* adrenergic stimulation could downregulate Dhrs7c expression. Finally, we confirmed that expression was also downregulated in two different models of failure and importantly, also in biopsies from human heart failure patients. **Conclusion** Our results show that the expression of Dhrs7c, a novel endo/sarcoplasmatic reticulum localized SDR, is inversely correlated with adrenergic stimulation and heart failure development.

Introduction

Heart failure (HF) is the final common pathway of most cardiovascular diseases. HF develops gradually as a result of pathological stress factors acting on the heart of which the most common causes are myocardial infarction (MI) and hypertension. Sustained myocardial stress is associated with increased ventricle wall stress and causes the heart to react with a compensatory program involving fibrosis [1] and cardiomyocyte growth (hypertrophy) [2, 3]. Although initially this is believed to be beneficial, on the long term this leads to cardiac remodelling and decompensation, resulting in decreased pump function and HF.

The course of HF development is inherently complex and involves multiple factors, including biomechanical, (neuro)hormonal and para- and autocrine activation. Neurohumoral factors including, catecholamines (adrenaline, noraderenaline), endothelin-1, angiotensin II and IGF-I have all been shown to generate cardiomyocyte hypertrophy in vitro [3]. This suggests that these factors activate overlapping gene programs, even though they activate different cellular pathways and have different effects on cardiomyocyte function. Examples of HF markers that are upregulated by these pathways are atrial and brain natriuretic peptides (*ANP* and *BNP*). These factors belong to the so called fetal gene program and have clinical relevance [4, 5]. Identification of additional genes controlled by these pathways would therefore be of interest since their expression might be associated to hypertrophy and/or HF development.

Here we performed gene expression screens on cultured rat cardiomyocytes activated with adrenergic stimuli phenylephrine (α-adrenergic) and isoproterenol (β-adrenergic). The combined analysis revealed a novel gene, *DHRS7c*, encoding a short chain dehydrogenase/reductase (SDR), which is downregulated by adrenergic stimulation both *in vitro* and *in vivo*. Moreover, we confirm the down-regulation in multiple rat HF models and in human HF patient biopsies.

Materials and Methods

Reagents and chemicals

Polyclonal anti-Dhrs7c antibody was generated as described in the supplemental materials and methods. Mouse monoclonal anti-α-actinin antibody EA53 was from Sigma-Aldrich Chemie B.V.. Rabbit polyclonal anti-troponin (ab9332) and anti-myc antibody were from Abcam (Cambridge, MA, USA). Rhodamine-phalloidin, Mitotracker red FM, Complex III subunit core 2 mouse monoclonal antibody and TO-PRO-3 were from Invitrogen. Mouse

monoclonal myc 9E10 and anti-GAPDH were from Santa cruz, mouse monoclonal anti-KDEL was from Stressgen. Goat anti-GM130 was a kind gift from Prof. F. Barr. L-[4,5-^{3}H]Leucine (37 MBq/ml; 5.85 TBq/mmol) was from GE Healthcare Europe (Diemen, Belgium). Unless otherwise stated all chemicals were purchased at the highest possible grade from Sigma-Aldrich Chemie B.V. (Zwijndrecht, The Netherlands).

Neonatal rat cardiac myocyte and fibroblast isolation

Neonatal rat ventricle cardiomyocytes (NRVCs) were isolated from the cardiac ventricles of one to three days old Sprague-Dawley (SD) pups after decapitation, as previously described [6]. NRVCs were cultured at 37ºC under 5% CO_2 in DMEM medium, supplemented with 10% FCS and penicillin-streptomycin (100 IU/ml and 100 µg/ml, respectively), as described previously [6]. For experiments in which cells were treated with hypertrophy inducing agents cells were first serum starved for 24 hours. Cells were then treated 24 hours with either phenylephrine (PE), isoproterenol (Iso) or endothelin-1 (ET-1).

Animal experiments

Continuous Iso/PE infusion (20mg/kg/day Iso and 20 mg/kg/day PE) was performed with C57Bl/6j mice of 3 to 4 months old. As a control saline infusion was used. Infusion was mediated using osmotic minipumps (Alzet, Maastricht, The Netherlands, Model 2002), which were inserted subcutaneously in the left flank of the mice. Mice were anesthetized with isoflurane (2% in O_2). Mice were sacrificed 2 or 7 days after insertion of the pumps and bodyweight and heart weight were determined. As a HF model we used male TGR(mRen2)27 (Ren2) rats that were homozygous for the expression of the mouse renin (Ren)-2 transgene (Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany) [7, 8]. SD rats were used as controls. Animals were fed *ad libitum*. Ren2 rats and control rats were sacrificed at 13 weeks of age [9]. As a separate model, HF was induced in Munich Wistar Fromter (MWF) rats by permanent ligation of the left coronary artery to produce a myocardial infarction (MI). Anaesthetic agents used: isoflurane (5% induction, 2.5% maintenance with oxygen) and proper anaesthesia was confirmed by reflex analysis and by heart rate monitoring. Analgesics used: buprenorphine 0.04 mg/kg, every 8 hours for 3 days. Control MWF rats underwent a sham operation. Twelve weeks after MI animals were sacrificed. All animals were sacrificed under anaesthesia, as described above, by heart removal. Myocardial tissues from all groups were snap frozen for molecular analyses. All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee for Animal Experiments of the University of Groningen.

[^{3}H]-Leucine incorporation assay

Cells were grown in 12-well plates (Greiner Bio-One) and subsequently starved for 24 hours in DMEM containing starvation medium lacking FCS. Subsequently, L-[4,5-^{3}H]Leucine was added to all wells either in the presence or absence of 50 µM PE, 10 µM Iso or 10 nM ET-1. Cells were cultured for an additional 20 hours and radioactivity incorporation was determined as described before [6].

Microarray Sample Preparation and Analysis

Total RNA from the cells or tissue was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). RNA quality was checked using the Agilent 2100 Bioanalyzer™ and subsequently prepared for microarray analysis using the standard Illumina® TotalPrep™-96 RNA Amplification Protocol (Ambion, Inc.) Samples were exponentially amplified from a starting amount of 50 ng to a final amount of 1 µg purified biotin-labeled cRNA using the Illumina® TotalPrep™-96 RNA Amplification Kit (Ambion, Inc.). This final cRNA was evaluated and the quality, concentration and size of the reaction productions were measured using the Agilent RNA 6000 Nano Kit (Agilent). Illumina RatRef-12 Beadchips were used for microarray analysis. Chips were scanned using the BeadXpress Reader™ (Illumina). Beadstudio™ Illumina was used to import the raw data and remove any background noise. Data was converted to standard format and exported for use in Agilent Genespring GX™ (version 11.0, Agilent). We used Genespring to perform quantile normalization on each individual well and further analysis of the data.

Cloning and adenoviral production

Myc-tagged Dhrs7c adenovirus was constructed as described in the supplementary methods. Each NRVC culture was infected at an MOI of 50-100. Medium was changed 13 hours after infection. Microscopy were performed 24 hours after infection.

Patients

Patients were described previously [10]. All patients had idiopathic dilated cardiomyopathy (DCM) with New York Heart Association (NYHA) class III-IV symptoms. Tissues from patients were obtained during cardiac catheterization by right ventricular endomyocardial biopsy. Control myocardial tissue from brain dead donors was harvested from non-failing, normal hearts which became available for cardiac transplantation. This protocol was approved by a local Ethics committee and all investigations were performed in accordance with the principles of the Declaration of Helsinki.

Quantitative real-time PCR, Western blotting and microscopy

Standard methodology was applied see supplemental methods.

Statistical analysis

All data are expressed as mean±SEM. The differences between two groups were determined by a Student's *t* test. Comparisons between more than two groups data were assessed by One-Way ANOVA followed by Bonferroni's post-hoc test. A value of $P<0.05$ was considered significant.

Results

Identification of *DHRS7c* by expression profiling

Using gene arrays we investigated differential gene expression in cultured primary neonatal rat cardiomyocytes that were stimulated for 24 hours with hypertrophy inducing agents phenylephrine (PE, α-adrenergic) and isoproterenol (Iso, β-adrenergic). This revealed 43 differentially expressed genes common to both stimuli (SFigure 1A). Interestingly, the strongest downregulated gene appeared to be a gene with unknown function. This gene annotated as *DHRS7c* was downregulated about 20 fold in Iso treated cardiomyocytes and more than 4 fold in PE treated cells. Bioinformatics (NCBI Blast analysis) revealed that *DHRS7c* is a member of the classical short chain dehydrogenase/reductase (SDR) family [11] and is conserved from fish to men (SFigure 1B).

Dhrs7c localizes to the endo/sarcoplasmatic reticulum

Based on the presence of an N-terminal signal sequence in Dhrs7c, this protein is predicted to localize to membranes. To investigate its particular localization, we made an adenoviral construct with Dhrs7c containing a C-terminal myc-tag. This protein was expressed in neonatal rat cardiomyocytes and its localization was determined by confocal immuno-fluorescence microscopy. As shown in Figure 1A, myc-tagged Dhrs7c did not appear to localize to the plasma membrane, but was visible in intracellular structures, predominantly around the nucleus. The identity of these cells as cardiomyocytes was confirmed by α-actinin staining (SFigure 2). Co-staining with the golgi marker GM130 or with mitotracker, a mitochondrial marker, did show no or only partial (golgi) co-localization (Figure 1B). Co-localization was, however, clearly observed with the endo/sarcoplasmatic reticulum (ER) marker KDEL, indicating that Dhrs7c is a novel ER protein (Figure 1B). To confirm that

endogenous Dhrs7c also localized to the SR we performed subcellular fractionation of a rat heart. Using a self-generated polyclonal against Dhrs7c we could show that Dhrs7c protein was enriched in the microsomal/ER fraction in Figure 1C. Together, these results indicate that Dhrs7c is an ER associated protein in cardiomyocytes.

Dhrs7c is not downregulated by ET-1 and hence this does not appear to be essential for hypertrophy development

Next we wanted to confirm the potential relation between Dhrs7c downregulation, adrenergic stimulation and hypertrophy development. In addition to PE and Iso stimulation, hypertrophy was also induced by endothelin I (ET-1). Hypertrophy induction in these cells was determined

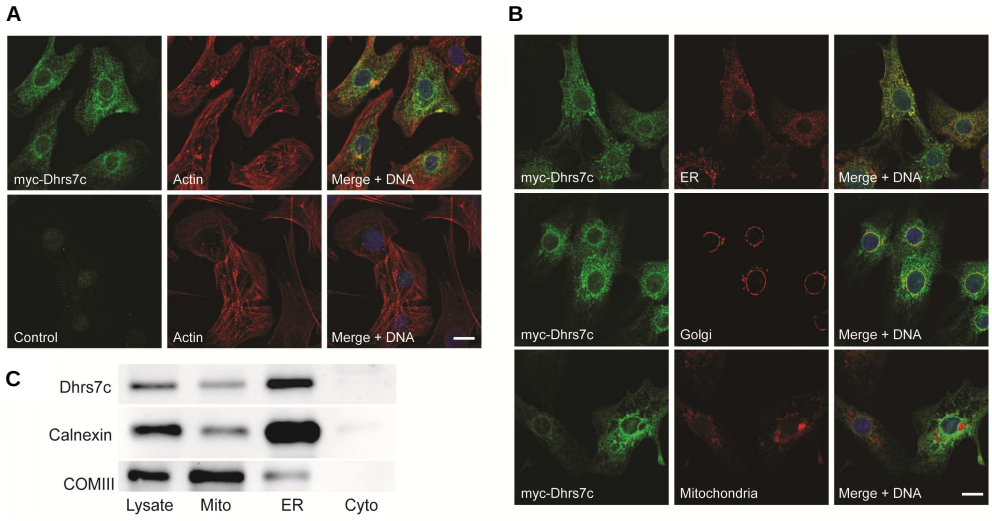


Figure 1. Dhrs7c is an endo/sarcoplasmatic reticulum localized protein

NRVCs were cultured *in vitro* and infected with recombinant adenovirus expressing myc-tagged Dhrs7c. A. After fixation and permeabilization myc-Dhrs7c cells were stained with anti-myc (green) for Dhrs7c and phalloidin (red) to stain the actin cytoskeleton. The merged picture at the right also contains TO-PRO-3 stained nuclei (blue). Top row shows myc-Dhrs7c infected cells. Bottom row shows control infected cells (showing faint non specific nuclear staining). B. Cells were stained with anti-myc (green) and anti-KDEL (red) (top row), anti-myc (green) and anti-GM130 (red) (middle row) and anti-myc (green) and mitotracker (red) (bottom row). The merged pictures at the right with TO-PRO-3 stained. C. Different cellular fractions of a rat heart were isolated and probed by Western blotting with anti-Dhrs7c, anti-Calnectin (ER marker) and anti-ComplexIII subunit Core2 (ComIII, mitochondrial marker). Mito=mitochondrial, ER=endo/sarcoplasmatic reticulum, and Cyto=cytosolic fraction. Bars indicate 10 µm.

by ^{3}H-Leucine incorporation, showing that PE and ET-1 strongly induced hypertrophy and that Iso gave a modest but significant induction of hypertrophy (Figure 2A). *ANP* expression showed the same pattern as ^{3}H-Leucine incorporation (Figure 2B). DHRS7c mRNA expression, however, showed a different pattern, with the strongest downregulation in isoproterenol treated cells (~20 fold), followed by PE treated cells (~5 fold), whereas in ET-1 treated cells the expression remained nearly constant (Figure 2C). At the protein level Dhrs7c could be detected in control and ET-1 treated cultures as a 34kda band, the predicted size of Dhrs7c (Figure 2D). This band was nearly absent in Iso and PE treated cultures (Figure 2D). Thus, Dhrs7c protein levels paralleled mRNA expression. Further investigation showed that the downregulation was concentration dependent with half maximal repression at 0.07 μM Iso and 12.2 μM PE (SFigure 3). Combined treatment with both compounds provided an additive effect with maximal downregulation and also dobutamine (β1-adrenergic) and clenbuterol (β2-adrenergic) provided clear downregulation, in contrast to tyramine, a non-adrenergic monoamine (SFigure 3 and data not shown). Direct activation of the downstream signaling components of the α- and β- adrenergic pathway, using PMA (activates PKC) and forskolin (activates adenylate cyclase), respectively, also resulted in profound repression of Dhrs7c (Figure 2E and 2F).

We also investigated the effect of myc-tagged Dhrs7c overexpression in isolated neonatal cardiomyocytes. Although no apparent phenotype was observed under standard culturing conditions, profound cell death was observed after Iso treatment, but not with ET-1 (Figure 2G). Overexpression was confirmed by Western blotting and concomittant siRNA could suppress overexpression and cell death, indicating that this is a Dhrs7c specific effect (SFigure 4). No increased caspase 3 cleavage was observed, suggesting that cell death was not mediated via apoptosis (data not shown).

Dhrs7c is a vertebrate SDR with a heart, muscle and skin specific expression

To further characterize *DHRS7c*, we next analyzed its tissue specific expression. RNA was isolated from different tissues of mouse and used for real-time PCR quantification of *DHRS7c* expression. As shown in Figure 3A, *DHRS7c* mRNA appeared to be highly expressed in heart and muscle, lower expression was observed in skin and in other tissues expression was virtually absent. At the protein level Dhrs7c was expressed in heart, muscle and skin, but not in other tissues (Figure 3B). This parallels our mRNA expression data; although in skin the RNA levels were somewhat lower as compared to heart and muscle. Similar results were obtained with rat tissues indicating that this specific expression is a conserved feature, at

least in mammals (SFigure 5). Since cardiac tissue consists predominantly of cardiomyocytes and fibroblasts, we also separated cardiomyocytes and fibroblasts from neonatal rat hearts to analyse cell type specific expression. As shown in Figure 3C and 3D,

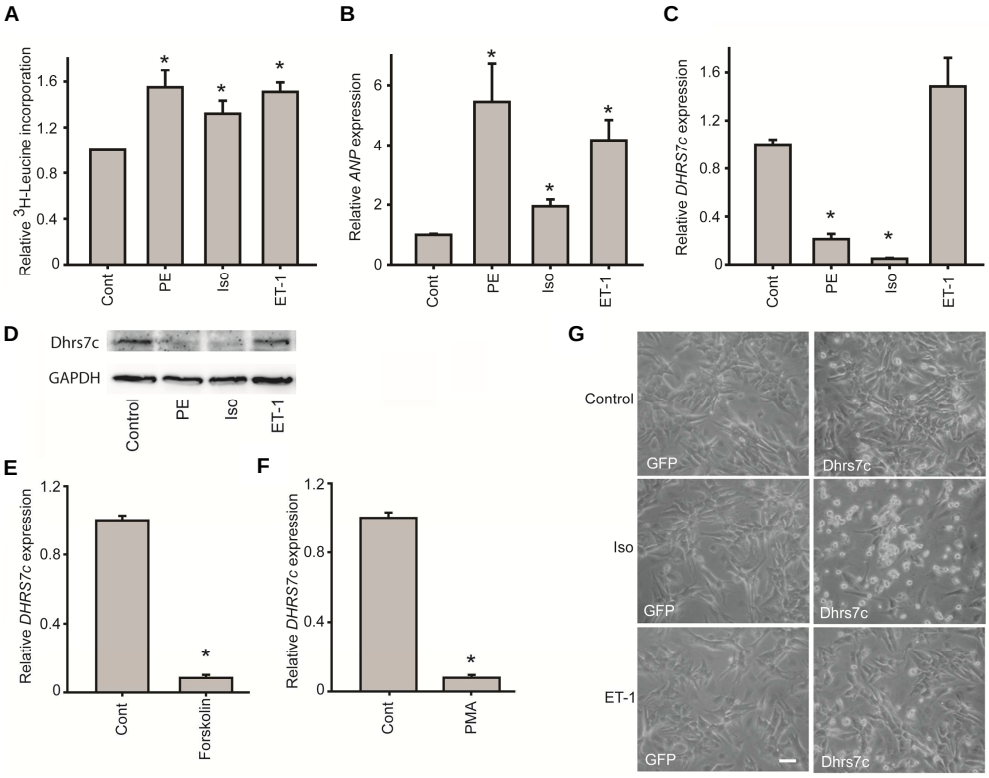


Figure 2. Adrenergic stimulation downregulates Dhrs7c expression in neonatal rat cardiomyocytes *in vitro*.

NRVCs were cultured for 24 hours in serum free medium and subsequently stimulated with PE, Iso or ET-1. After 24 hours stimulation cells were harvested for analysis. A. [^{3}H]-Leucine incorporation in cellular protein was analysed to determine the hypertrophic effects of the different hormones. Values are relative to the respective non-treated cultures (n=3). B. *ANP* expression in the different cultures was determined by RT-PCR and corrected for *cyclophilin A* expression (n=9). C. *DHRS7c* expression in the different cultures was determined by RT-PCR and corrected for *cyclophilin A* expression (n=9). D. Representative Western blot of Dhrs7c protein expression in the different cultures. E. Similar as in C, but now Forskolin treated culteres were analysed (n=6). F. Similar as in C, but now PMA treated culteres were analysed (n=4). G. NRVCs were cultured *in vitro* and infected with recombinant adenovirus expressing myc-tagged Dhrs7c or as a control a GFP expressing virus. After 24 hours in serum free medium cells were either stimulated with Iso or ET-1 or left untreated for another 24 hours. Light microscopy pictures of myc-Dhrs7c expressing cells (top row) or control GFP transfected cells (bottom row) with the indicated treatments are shown. * indicates $p<0.05$ as compared to control.

Dhrs7c RNA and protein expression were predominantly confined to cardiomyocytes in the heart. During this analysis we also observed that Dhrs7c protein expression was higher in adult hearts as compared to neonatal hearts (Figure 3E).

Dhrs7c mRNA and protein level are downregulated in animal heart failure models

Based on the profound downregulation of Dhrs7c *in vitro* by adrenergic stimulation we next investigated if adrenergic stimulation could also downregulate Dhrs7c *in vivo*. *In vitro* a combination of Iso/PE gave a maximal downregulation within two days. Therefore a combination of Iso/PE (20 mg/kg/day each) was provided to mice using osmotic minipumps and after 2 and 7 days treatment these mice were sacrificed. Within 2 days heart weight to body weight (HW/BW) significantly increased (from 4.75±0.16 mg/g to 6.14±0.43 mg/g,

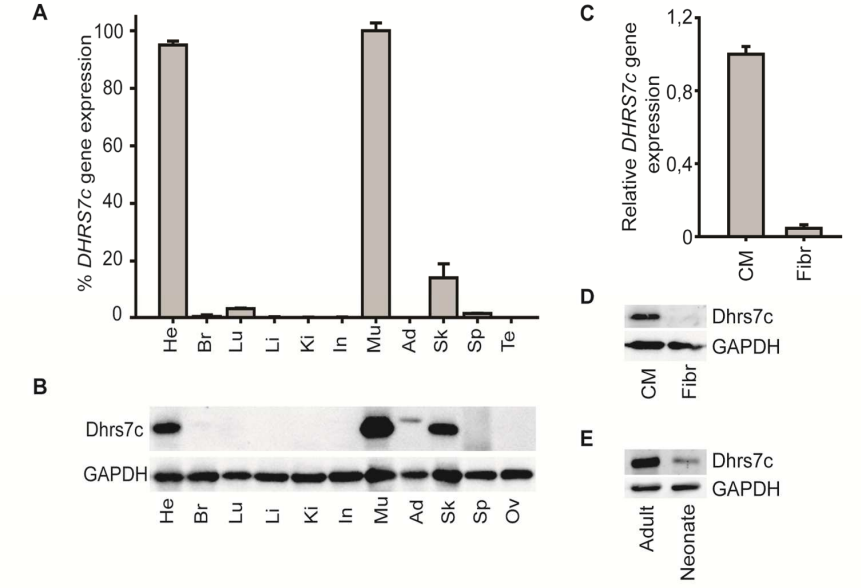


Figure 3. *DHRS7c* mRNA and protein expression is restricted to heart, muscle and skin.
RNA and proteins were isolated from the indicated mouse organs, tissues or cells and used for RT-PCR or Western blot analysis. A. Bar diagram showing relative *DHRS7c* mRNA expression in different mouse tissues. Levels were measured in triplicate and corrected for *GAPDH*. B. Western blot analysis of Dhrs7c in different mouse tissues. As a loading control GAPDH is shown. C. *DHRS7c* mRNA in NRVCs and cardiac fibroblasts (n=4) corrected for *cyclophilin A*. D. Dhrs7c protein expression in NRVCs and cardiac fibroblasts. E. Dhrs7c protein expression in neonatal versus adult rat hearts. Abbreviations: He=Heart, Br=Brain, Lu=Lung, Li=Liver, Ki=Kidney, In=Intestine, Mu=Muscle, Ad=Adipose, Sk=skin, Sp=Spleen, Te=Testis, Ov=Ovary, CM=cardiomyo-cytes, Fibr=fibroblasts.

p<0.05) as a result of cardiac hypertrophy development and also BNP expression was significantly induced (from 1.07±0.34 to 4.86±1.00, P<0.05). In these hearts Dhrs7c downregulation could be observed at the RNA and the protein level, although the latter was less pronounced (Figure 4A). After 7 days treatment these effects were, however, less clear and not significant at the protein level (Figure 4B), suggesting a temporal effect.

Dhrs7c is downregulated in animal HF models

Since adrenergic infusion is a rather artificial model, we were wondering whether Dhrs7c would also be downregulated in other disease models. In particular we analysed downregulation in Ren2 rats that overexpress mouse renin, generating hypertension resulting in HF development [7, 9]. In Ren2 rats HF development was evident from the increased HW/BW ratio as compared to control animals (5.40±0.20 mg/g vs 3.30±0.08 mg/g, p<0.05). Moreover, in these Ren2 animals cardiac ANP expression, a marker for HF development, was strongly increased (12.18±0.59 fold, p<0.05). *DHRS7c* gene expression was downregulated in Ren2

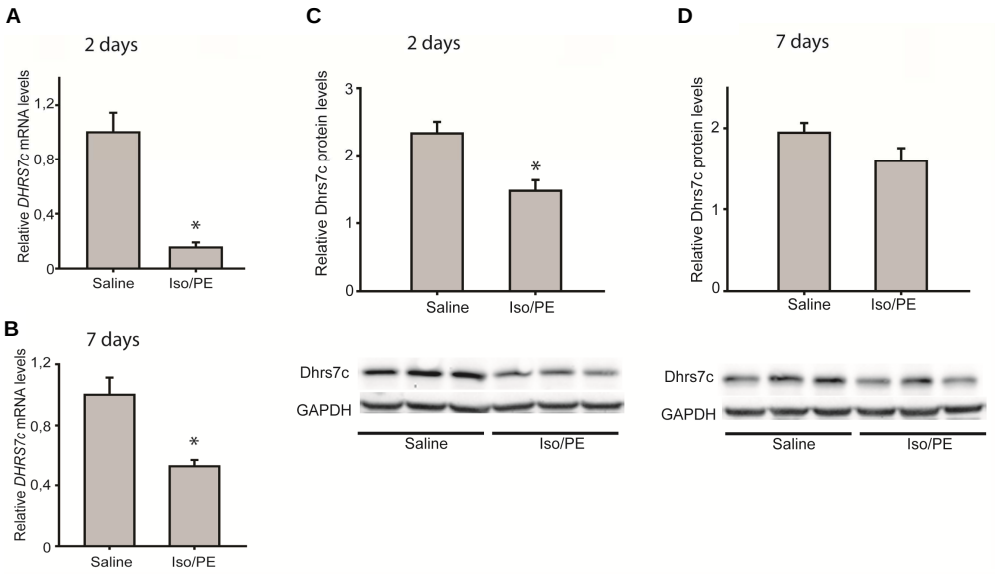


Figure 4. *DHRS7c* mRNA and protein expression are downregulated after adrenergic stimulation *in vivo*. Mice obtained a contiuous Iso/PE infusion (20 mg/kg/day of each) using osmotic minipumps for 2 or 7 days. Cardiac gene and protein expression of Dhrs7c were subsequently determined. A. Cardiac DHRS7c gene expression after 2 days Iso/PE infusion (n=3 control, n=5 Iso/PE). B Same as A, but after 7 days (n=6 control, n=3 Iso/PE). C. Dhrs7c protein expression after 2 days Iso/PE infusion (n=7 control, n=5 Iso/PE). D Same as C, but after 7 days (n=6 control, n=4 Iso/PE). * indiates p<0.05 as compared to the control.

animals and this was also true for the Dhrs7c protein levels (Figure 5A). Since myocardial infarction (MI) is one of the major causes of HF development we also analyzed Dhrs7c expression in a post-MI rat HF model. These animals had a mean infarct size of 46.9±2.5 %. Hypertrophy development was evident from the increased HW/BW ratio (5.30±0.30 mg/g vs 3.41±0.10 mg/g in sham, $p<0.05$) and ANP expression was elevated (14.73±0.40 fold, $p<0.05$). Dhrs7c RNA and protein levels were significantly down regulated in the non-infarcted hypertrophied areas in these post-MI animals, as compared to sham operated animals (Figure 5B).

Dhrs7c protein level is downregulated in heart failure patients

Finally, we investigated Dhrs7c protein expression in heart biopsies of patients with severe DCM and ischemic HF (NYHA class III and IV) [10]. As compared to control biopsies, the expression of Dhrs7c was significantly downregulated in these patients (Figure 5C). Together, this indicates that Dhrs7c expression in the heart is inversely associated with HF development.

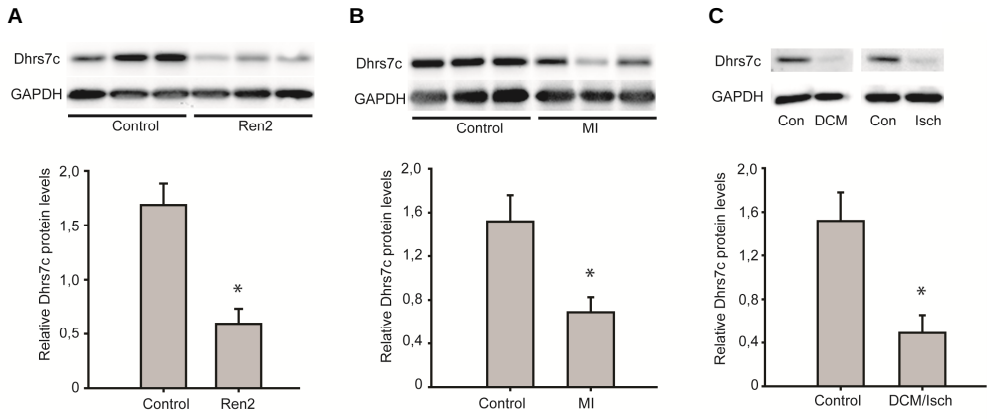


Figure 5. Dhrs7c is downregulated during heart failure.

RNA and/or protein were isolated from heart tissue and used for RT-PCR or Western blot analysis. A. RT-PCR on cDNA from Ren2 (n=7) and control SD (n=6) rats. *ANP* (top panel) and *DHRS7c* (lower panel) expression is shown relative to *GAPDH* levels. B. Western blot analysis of Dhrs7c expression in the same Ren2 and SD heart samples. Representative blots are shown together with the quantified data (lower panel). C. Western blot analysis of heart tissue from post-MI HF and sham operated MWF. Representative blots are shown together with the quantified data (lower panel) (n=5 for both groups). D. Dhrs7c protein expression in heart biopsies from DCM (n=3) or ischemic heart disease (n=2) patients and as a control hearts from persons without heart disease (n=6). Representative blots are shown and the bottom panel shows the quantified data. * indicates $p<0.05$ as compared to control. MI=post-MI HF, DCM=dilated cardiomyopathy, Isch=ischemic heart disease.

Discussion

In the present paper we describe the identification of a novel SDR, termed Dhrs7c, which is down-regulated in response to adrenergic stimulation. Dhrs7c is conserved in vertebrates and its restricted expression in heart, muscle and skin, suggests that it has tissue specific functions. In two HF models in rats with different etiology, Dhrs7c was downregulated at both the RNA and protein level. Importantly, downregulation was observed also in human heart biopsies of patients with DCM and ischemic HF.

Using gene arrays we uncovered a novel short chain dehydrogenase/reductase, termed Dhrs7c, which was downregulated in cardiomyocytes after Iso and PE stimulation. Dhrs7c contains an N-terminal transmembrane domain and our microscopical analysis and subcellular fractionation showed that Dhrs7c localized to the endo/sarcoplasmatic reticulum in cardiomyocytes. This is common for many of the other SDR family members and also its closest homolog Dhrs7b has been suggested to localize to the endoplasmatic reticulum [12] .

In vitro, Dhrs7c expression was downregulated upon Iso and PE treatment (β- and α-adrenergic agonist respectively), but surprisingly not after ET-1 treatment, even though ET-1 strongly induced hypertrophy and *ANP* expression, similar to PE. This indicates that Dhrs7c downregulation is not directly linked to hypertrophy and, moreover, that its downregulation is not a prerequisite for hypertrophy development. In contrast, Iso treatment only generated modest hypertrophy, but provided the strongest downregulation of Dhrs7c. Together, this suggests that downregulation might be linked to adrenergic stimulation, rather than to hypertrophy. This is further supported by the observations that activation of the downstream intracellular targets of these adrenergic pathways, adenylate cyclase (β-adrenergic) and PKC (α-adrenergic), with respectively forskolin and PMA, also resulted in downregulation of *DHRS7c* expression. Moreover, other adrenergic stimuli like dobutamin (β1-receptor) and clenbuterol (β2-receptor) also generated profound downregulation, whereas tyramine, a monoamine that does not activate adrenergic pathways, did not generate any downregulation.

Also *in vivo* Dhrs7c was downregulated by adrenergic stimulation, although this appeared to be a temporal effect. Two days after continued infusion of Iso/PE downregulation was stronger as compared to 7 days infusion. One possible explanation could be desensitizing of β-adrenergic signaling in the adult heart. This is a well-known phenomenon [13, 14] and also other genes like ICERI have shown to be temporal repressed in the heart by adrenergic stimulation [15]. Interestingly, in two independent HF models (hypertensive and post-MI) downregulation of Dhrs7c expression was observed, indicating

that this also occurs in more general cardiac disease models. Finally, downregulation was observed in human HF patients, supporting the generalizability of our experimental findings and indicating that this might have clinical relevance, too. Whether the decrease in Dhrs7c in rat and human HF are associated with adrenergic signaling is not known, but moderate adrenergic stimulation may occur in our Ren2 transgenic model and post-MI HF [16] and enhanced adrenergic signaling in human HF is a hallmark of this disease. We also observed that *in vitro* stretching of cardiomyocytes could downregulate Dhrs7c expression, indicating that the regulation of Dhrs7c might be more complex and does not only depend on adrenergic signaling (data not shown).

The identification of the regulatory transcription factors will be pivotal in understanding the regulation of Dhrs7c, but equally important will be the clarification of its biological function. So far, the only observed phenotype was cell death in overexpressing Dhrs7c cardiomyocytes treated with Iso. This indicates that downregulation could be important during adrenergic stimulation. The cause of cell death is not clear yet, but did not appear to be apoptotic. Although this suggests a prominent function for this dehydrogenase/reductase, it does not clarify its biological function. Most classic SDR family members, to which also Dhrs7c belongs, and which localize to the ER, are involved in steroid/sterol biosynthesis [11]. Although heart specific steroid synthesis seems peculiar we like to note that recently also another heart specific gene, *SRD5A2L2*, potentially implicated in steroid synthesis has been identified [17]. Nevertheless, other ER like functions cannot be excluded and identification of its substrates will be crucial in clarifying its function.

In conclusion, using gene arrays we uncovered a novel short chain dehydrogenase/reductase that is downregulated in cardiomyocytes upon adrenergic stimulation and in experimental HF models. Our observation that also in human HF patients Dhrs7c expression is downregulated, clearly warrants further research in the regulation and function of this gene. We foresee that our recently initiated combined approach of biochemical and transgenic animal studies will soon shed light on the function of this intriguing protein in HF.

Acknowledgements

We thank Silke Maass, Linda van Genne and Maarten Zwartbol for their expert technical assistance. Cardiac samples of MWF rats were kindly provided by Mariusz Szymanski. The gene array was performed at the Department of Genetics, University Medical Center Groningen, The Netherlands.

References

1. van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J. Myocardial remodeling after infarction: the role of myofibroblasts. *Nat Rev Cardiol* 2010;7:30-37.

2. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. *Annu Rev Physiol* 2003;65:45-79.

3. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006;7:589-600.

4. Kjaer A, Hesse B. Heart failure and neuroendocrine activation: diagnostic, prognostic and therapeutic perspectives. *Clin Physiol* 2001;21:661-672.

5. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail Rev* 2007;12:331-343.

6. Lu B, Mahmud H, Maass AH, Yu B, van Gilst WH, de Boer RA, Sillje HH. The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy in vitro. *PLoS One* 2010;5:e12963.

7. de Boer RA, Pokharel S, Flesch M, van Kampen DA, Suurmeijer AJ, Boomsma F, van Gilst WH, van Veldhuisen DJ, Pinto YM. Extracellular signal regulated kinase and SMAD signaling both mediate the angiotensin II driven progression towards overt heart failure in homozygous TGR(mRen2)27. *J Mol Med* 2004;82:678-687.

8. Lee MA, Bohm M, Paul M, Bader M, Ganten U, Ganten D. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. *Am J Physiol* 1996;270:E919-E929.

9. Ruifrok WP, Qian C, Sillje HH, van Goor H, van Veldhuisen DJ, van Gilst WH, de Boer RA. Heart failure-associated anemia: bone marrow dysfunction and response to erythropoietin. *J Mol Med* 2010;

10. de Boer RA, Henning RH, Suurmeijer AJ, Pinto YM, Olthof E, Kirkels JH, van Gilst WH, Crijns HJ, van Veldhuisen DJ. Early expression of natriuretic peptides and SERCA in mild heart failure: association with severity of the disease. *Int J Cardiol* 2001;78:5-12.

11. Bray JE, Marsden BD, Oppermann U. The human short-chain dehydrogenase/reductase (SDR) superfamily: a bioinformatics summary. *Chem Biol Interact* 2009;178:99-109.

12. Keller B, Meier M, Adamski J. Comparison of predicted and experimental subcellular localization of two putative rat steroid dehydrogenases from the short-chain dehydrogenase/reductase protein superfamily. *Mol Cell Endocrinol* 2009;301:43-46.

13. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *N Engl J Med* 1982;307:205-211.

14. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res* 2003;93:896-906.

15. Tomita H, Nazmy M, Kajimoto K, Yehia G, Molina CA, Sadoshima J. Inducible cAMP early repressor (ICER) is a negative-feedback regulator of cardiac hypertrophy and an important mediator of cardiac myocyte apoptosis in response to beta-adrenergic receptor stimulation. *Circ Res* 2003;93:12-22.

16. Teisman AC, Pinto YM, Buikema H, Flesch M, Bohm M, Paul M, van Gilst WH. Dissociation of blood pressure reduction from end-organ damage in TGR(mREN2)27 transgenic hypertensive rats. *J Hypertens* 1998;16:1759-1765.

17. Beqqali A, Kloots J, Ward-van Oostwaard, Mummery C, Passier R. Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. *Stem Cells* 2006;24:1956-1967.

Supplemental Materials and Methods

Production of recombinant adenovirus

To generate recombinant adenoviral vectors the ViraPower Adenovirus Expression System (Invitrogen) was used according to the manufacturer's instructions. Full length mouse *DHRS7c* was first cloned into pcDNA4/TOP/myc-HisC,creating a C-terminal fusion with the myc tag. The whole expression cassette containing cytomegalovirus (CMV) promoter, *DHRS7c*-myc and BGH pA sequences was transferred to the shuttle vector pENTR4. Using LR clonase (Invitrogen) the whole cassette was subsequently recombined into the pAD/PL-DEST adenoviral vector. For siRNA, the shuttle vector pENTR4/H1 was constructed by inserting the H1 promoter from the pTER+ siRNA vector. In addition an EGFP expression cassette was cloned into this vector in order to visualize infected cells. *DHRS7c* specific siRNA targeting oligo nucleotides and as a control GL2 (luciferase) oligo nucleotides were cloned into this vector. Subsequent recombination into pAD/PL-DEST adenoviral vector was performed. All constructs were verified by sequencing. Recombinant adenovirus was produced by transfecting adenoviral constructs into HEK 293A cells using Lipofectamine 2000 (Invitrogen). The titer of adenovirus was assessed by the limiting-dilution method (Clontech Adenovirus rapid titer manual) using $5x10^5$ cells per well HEK 293A cells plated on 12-well plates (Greiner Bio-One).

Production of polyclonal antibodies

Polyclonal rabbit antiserum specific for Dhrs7c was generated against the unique C-terminal domain of Dhrs7c. A hexahistidine (His6) - tagged C-terminal fragment of mouse Dhrs7c (a.a. 212-311) was expressed in *Escherichia coli* using the pQE30 expression vector (Qiagen) and subsequently purified using NiNTA agarose (Qiagen). This purified recombinant protein fragment was used for immunization of rabbits (Biogenes GmbH, Berlin, Germany). Dhrs7c specific antibody was isolated from the rabbit serum using the same recombinant Dhrs7c protein fragment covalently coupled to AminoLink Plus beads (Thermo Scientific). For Western blotting this purified antibody was used at a concentration of 0.5 µg/ml.

Microscopy

Cells were grown on coverslips coated with 1 $\mu g/cm^2$ laminin (Millipore) for the indicated time periods and subsequently washed with PBS and fixed in paraformaldehyde solution for 10 min at room temperature. After permeabilization with 0.5% Triton X100, antibody incubations were performed in PBS+1%BSA for 1 hour at room temperature. Primary antibodies used

were: mouse monoclonal anti-KDEL (1:100), anti-myc 9E10 (1:100), goat anti-GM130 (1:1000) and rabbit anti-myc (1:2000). Secondary Alexa antibodies (Invitrogen, The Netherlands) were used at 2 µg/ml. Nuclei were stained with TO-PRO3. For mitotracker staining cells were incubated for 30 minutes with 250 nM mitotracker Red FM and subsequently fixed and processed as described above. Confocal images were made on a Leica SP2 AOBS confocal microscope. All images were processed with ImageJ 1.43M (NIH, USA) and Adobe Photoshop 7.01. Z-stack projections of the confocal images are shown.

Quantitative Real-Time PCR

Relative gene expression of *DHRS7c*, *ANP*, *BNP, cyclophilin A* and *GAPDH* was determined by quantitative real time PCR (RT-PCR) on a Bio-Rad CFX384 real time system using SYBR Green dye. Gene expression was determined by correcting for reference gene values (*cyclophilin A* or *GAPDH*), and the calculated values were expressed relative to the control group per experiment. Primer sequences can be found in Table S1.

Western blotting

NRVCs and tissues were homogenized in ice-cold RIPA (50 mM Tris pH 8.0, 1% nonidet P40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl) containing phosphatase inhibitor cocktail 1 (Sigma), protease inhibitor (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 15 mM NaVanadate for 30 min. Equal amounts of proteins were loaded on 12% polycrylamide gels. After electrophoresis, the gels were blotted onto nitrocellulose membranes. Membranes were then incubated with primary antibodies overnight at 4° C and signals were visualized with ECL and analyzed with densitometry (Syngene, Cambridge, United Kingdom).

Table S1

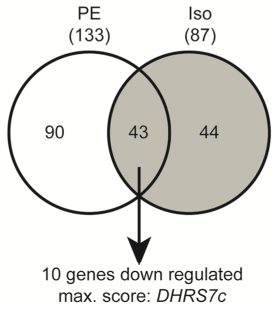
Primers for real time PCR

Name	Species	Sequence
DHRS7c	mouse	Forward: GGAGAACAGGCCAGATTGTG
		Reverse: CTCGGAGGCAGTCAAAGAAG
	rat	Forward: GGACGCCAACTACTTTGGAC
		Reverse: GTCGAAGAAGCCCATGACAG
GAPDH	mouse / rat	Forward: CATCAAGAAGGTGGTGAAGC
		Reverse: ACCACCCTGTTGCTGTAG
Cycolophilin A	mouse / rat	Forward: CCTCATACCAGCGACGATTC
		Reverse: ATGTGGAGGAGTCTCACTTC

Primers for generation of recombinant adenoviruses

cDNA primers		
DHRS7c	Forward	GAAGGATCCGTCGACATGGGGCTCATGGCTGTCCTG
	Reverse	GAACTCGAGCCCGGGCACCCTCTTCTGGGACATTGAGC
siRNA primers		
GL2	Forward	GATCCCCGTACGCGGAATACTTCGATTCAAGAGATCGAAGT ATTCCGCGTACGTTTTTGGAAA
	Reverse	AGCTTTTCCAAAAACGTACGCGGAATACTTCGATCTCTTGA ATCGAAGTATTCCGCGTACGGG
DHRS7c	Forward	GATCCCAGGTTGAGGAATACGATGTTTCAAGAGAACATCGT ATTCCTCAACCTTTTTTGGAAA
	Reverse	AGCTTTTCCAAAAAAGGTTGAGGAATACGATGTTCTCTTGA AACATCGTATTCCTCAACCTGG

A

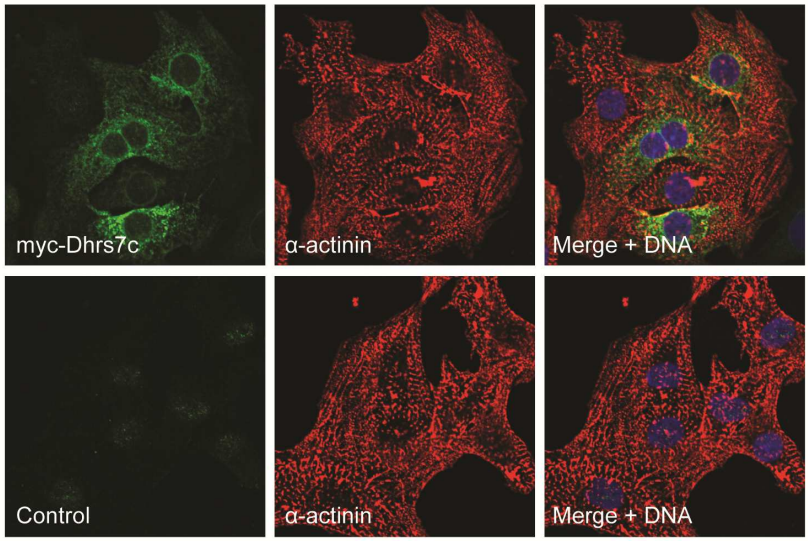


B

```
Hs  MGVMAMLMLPLLLLGISGLLFIYQEVSRLWSKSAVQNKVVVITDAISGLGKECARVFHTG 60
Rn  MGIMAVLMLPLLLLGVSGLLFIYQEASRLWSKSAVQNKVVVITDALSGLGKECARVFNAG 60
Gg  MGILAVFALPLLLLGISGIIYIYQTVIWLVSKSAVQNKVVVITDAISGLGKECSRVFHSG 60
Tn  ------MLLPLLIVVSAGVYYIYNEILLFMSKSLVRNKVVVITDAVSGVGTECSRLFHKG 54
          : ****::  :*: :**:    : *** *:*********:**:*.**:*:*: *

Hs  GARLVLCGKNWERLENLYDALISVADPSKTFTPKLVLLDLSDISCVPDVAKEVLDCYGCV 120
Rn  GARLVLCGKNWEGLESLYAALTSVADPSKTFTPKLVLLDLSDISCVEDVAKEVLDCYGCV 120
Gg  GARLVLCGRTWEKLEALYDALISVADPSTTYTPKLVLLDISDTDYIQDVAKEILNCYGSV 120
Tn  GARLILCGTSWDKLESLYDSLTNDADPKETFAPKLVLLDFSDMESMEDVVAEVVDCYGCV 114
    ****:*** .*: ** ** :* . ***. *::*******:** . : **. *:::***.*

Hs  DILINNASVKVKGPAHKISLELDKKIMDANYFGPITLTKALLPNMISRRTGQIVLVNNIQ 180
Rn  DILINNASVKVKGPAHKISLELDKKIMDANYFGPITFTKVLLPNMISRRTGQIVLVNNIQ 180
Gg  DILINNASMKVKGAVQSISLELDKKIMDANYFGPITLTKAILPNMISRRTGQIVLINSIQ 180
Tn  DVLICNSSMKLKAPVQNISLEIDRNIMDINYFGPSTLAKGVLPTMISRRSGHIILVNSIQ 174
    *:** *:*:*:*...:.****:*::*** ***** *::* :**.*****:*:*:*:.**

Hs  GKFGIPFRTT**YAAS**KHAALGFFDCLRAEVEEYDVVISTVSPTFIRSYHVYP--EQGNWEA 238
Rn  AKFGIPFRTA**YAAS**KHAVMGFFDCLRAEVEEYDVVVSTVSPTFIRSYQAYP--EQRNWGS 238
Gg  GKIGVPFRAA**YAAS**KHAAVGFFDCLRAEMEEFDISVSTVNPTFICSYHHQP--APGNWEA 238
Tn  GRLAVPFRSS**YAAS**KHAAQAFFDCLRAEVEEYGIVVSTISPTFINACEPTPVKEAASKPN 234
    .::.:***::*******. .********:**:.: :**:. *** : .  *     .

Hs  SIWKFFFRKLTYGVHPVEVAEEVMRTVRRKKQEVFMANPIPKAAVYVRTFFPEFFFAVVA 298
Rn  SICKFFCRKLTYGVHPVEVAEEVMRTVRRKKQEVFMANPVPKAAVFIRTFFPELFFAVVA 298
Gg  SIWKFFFRKVTYGVHPAEVAEEVLRTVSSKKQEVLMANPIPRAAVYIRTFFPEMFFAIVA 298
Tn  SLSTFIARQMSHGVRPSVLANEVMQTVNRKRKEVTMAHPIPRVALCLRSLLPSFLFAVLG 294
    *: .*: *::::**:*  :*:**::**  *::** **:*:*:.*: :*:::*.::**::.

Hs  CGVKEKLNVPEEG 311
Rn  CGVKEKLSVPEEG 311
Gg  SGIREKLKTEQEN 311
Tn  AGVKDSVL----- 302
```

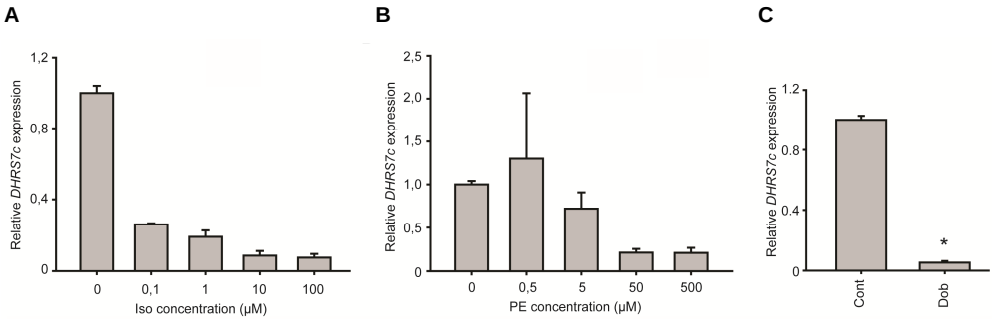
SFigure 1. Identification of *DHRS7c* in a combined gene array approach

A. Venn diagram indicating the numbers of genes found to be differentially regulated in primary cultured cardio-myocytes treated with PE and Iso as compared to untreated cultures. Only those genes with a fold change of 2 or higher and a p value lower than 0.05 were included (N=3, each). B. Alignment of human (Hs), rat (Rn), chicken (Gg) and pufferfish (Tn) Dhrs7c. Underlined is the transmembrane segment and the conserved catalytic site is indicated in bold.* identical residue; : highly similar residue; . similar residue.



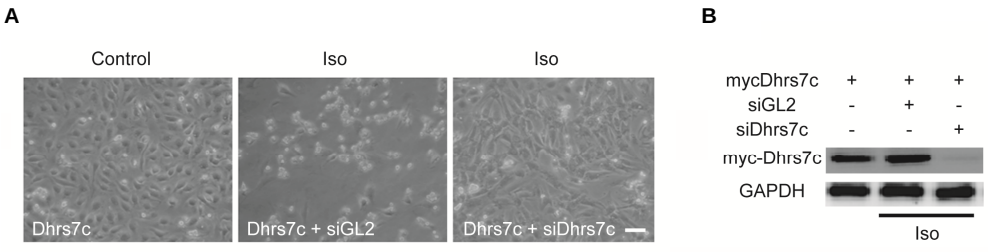
SFigure 2. myc-Dhrs7c expression in cardiomyocytes

Cardiomyocytes were cultured *in vitro* and infected with recombinant adenovirus expressing myc-tagged Dhrs7c. After fixation and permeabilization myc-Dhrs7c was stained with anti-myc (green) anti-α-actininin staining (red) was used to specifically identify cardiomyocytes. The merged picture at the right also contains TO-PRO-3 stained nuclei (blue).

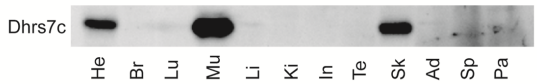


SFigure 3. Iso and PE concentration dependent downregulation of *DHRS7c* in neonatal rat cardiomyocytes *in vitro*

NRVCs were cultured for 24 hours in serum free medium and subsequently stimulated with Iso (A) or PE (B) at different concentrations. After 24 hours stimulation cells were harvested for analysis. *DHRS7c* expression in the different cultures was determined by RT-PCR and corrected for *cyclophilin A* expression (n=3). C. Same as above, but cells were now stimulated with 10 μM dobutamine.



SFigure 4. Rescue of cell death phenotype of overexpressed Dhrs7c by concomitant siRNA silencing
A. Light microscopy pictures of cells infected with myc-Dhrs7c expressing virus in the presence of siDhrs7c virus to silence *Dhrs7c* expression on in the presence of control siGL2 virus. Iso treatment is indicated. B. Western blot analysis of cell cultures treated with the different adenovirus combinations, showing downregulation of expression with siDhrs7c adenovirus only. Bars indicate 50 µm.



SFigure 5. *DHRS7c* mRNA and protein expression is restricted to heart, muscle and skin
Proteins were isolated from the indicated rat organs and tissues and used for Western blot analysis. Abbreviations: He=Heart, Br=Brain, Lu=Lung, Li=Liver, Ki=Kidney, In=Intestine, Mu=Muscle, Ad=Adipose, Sk=skin, Sp=Spleen, Te=Testis, Pa=pancreas.

Chapter 6

Exploring the function of Dhrs7c by cardiac-specific overexpression in mice

Bo Lu
Rudolf A. de Boer
Bart van de Sluis
Jan M. van Deursen
Wiek H. van Gilst
Herman H.W. Silljé

Manuscript in preparation

Abstract

The short chain dehydrogenase/reductase (SDR) family member 7C (Dhrs7c) is predominantly expressed in heart and muscle. We recently showed that Dhrs7c is downregulated in rodent heart failure (HF) models and, importantly, also in cardiac biopsies from human HF patients. Moreover, this downregulation appeared to be linked to adrenergic stimulation and *in vitro* overexpression of Dhrs7 appeared to be lethal upon adrenergic activation. So far the exact function of this novel SDR has, however, remained elusive. In order to investigate the *in vivo* role of Dhrs7c in the heart, we generated transgenic mice with cardiac specific Dhrs7c overexpression. A transgenic (TG) mouse line with approximately 5 fold overexpression was obtained. These mice behaved normal and did not show any obvious phenotype. Continuous adrenergic activation by infusion of isoproterenol and phenylephrine (Iso/PE) generated cardiac hypertrophy in these animals to a similar extend as compared to the not-transgenic (NTG) littermates. Also expression of the HF-marker ANP was similarly increased in both TG and NTG groups after Iso/PE infusion. Hemodynamic data was also not significantly different, although some trend was observed for a better fuction in (dPdtmin, dPdtmax, Tau g) in the TG group both at baseline and after Iso/PE infusion. Fibrotic scores were similar in TG and NTG groups, although we did observe a significantly increased expression of metalloproteinase II (MMP2) in the TG Iso/PE group as compared to the NTG Iso/PE group. This might suggest the presence of subtle differences in the extracellular matrix (ECM). Similarly Ca^{2+} handling might be somewhat altered in the TG Iso/PE treated group as compared to the NTG Iso/PE group, based on a significantly higher expression of the L-type Ca^{2+} channel. Thus although no major cardiac phenotype was observed, these small alterations might explain the slightly, albeit not significant, improved cardiac function in TG animals.

Introduction

Heart failure (HF) is the common end-stage of many cardiovascular diseases. It is defined as the inability of the heart to supply adequate blood in response to systemic demands. The incidence of HF is 2-3% in developed countries, and rises sharply to 10% in those aged 75 years and older [1]. Despite therapeutic improvements in the past decades, HF is still associated with a high mortality and morbidity. It will be pivotal to improve our knowledge on this complex disease in order to identify new treatment opportunities.

By whole genome gene expression analysis we recently identified a novel gene, termed Dhrs7c that showed heart failure associated gene expression [2]. In particular, expression was downregulated in several animal HF models as well as in cardiac biopsies of human HF patients. *In vitro* studies with cultured primary neonatal rat cardiomyocytes revealed that Dhrs7c was strongly downregulated by isoproterenol (Iso, β-adrenergic agonist) and moderately downregulated by stimulation with phenyl-ephrine (PE, α-adrenergic agonist). Endothelin-1 (ET-1) stimulation of rat neonatal cardiomyocytes did not result in Dhrs7c downregulation, despite hypertrophy development similar to PE. This indicates that Dhrs7c downregulation is not linked to hypertrophy development, but rather associated with adrenergic stimulation. Also *in vivo* Dhrs7c downregulation was observed in mice after infusion with Iso/PE, which is in agreement with the *in vitro* results. Interestingly, *in vitro* studies with Dhrs7c overexpression showed toxicity only in the presence of Iso, but not ET-1, suggesting that downregulation of Dhrs7c during adrenergic stimulation could be protective. Despite these observations the function of this predominantly heart and muscle expressed gene remained elusive.

To gain insights into the *in vivo* function of Dhrs7c in the heart, we constructed a transgenic mouse with cardiac-specific Dhrs7c overexpression. These transgenic mice showed increased Dhrs7c mRNA and protein expression in the heart only. Baseline cardiac parameters were determined as well as the effect of Iso/PE infusion on cardiac function.

Methods and materials

Transgenic animals

The mouse *DHRS7c* gene (GeneBank: NM_001013013) was amplified by polymerase chain reaction (PCR) using primers containing a SalI and HindIII restriction site, respectively. Forward primer: GAAGTCGACACCATGGGGCTCATGGCTGTCCTG; Reverse primer: GAAAAGCTTAGGTTAACCCTCTTCTGGGACATTGAGC. This PCR product was cloned into

a previously described vector containing the cardiac specific α-MHC promoter [3]. The BamHI fragment of this construct, containing α-MHC promoter and downstream Dhrs7c cDNA sequence, was subsequently used for pronuclear injections to generate Dhrs7c transgenic mice (FVB background). The transgenic mice were made by the UMCG mouse clinic (Marten Hofker) in collaboration with the Mayo Clinics. These transgenic mice were subsequently back crossed with C57BL/6 mice. In all experiments performed in this study, age- and sex-matched non-transgenic littermates were used for comparison with the Dhrs7c transgenic mice.

Groups and experimental protocols

All animal protocols were approved by the Animal Ethical Committee of the University of Groningen. A total of 40 mice, 20 transgenic (TG) and 20 non-transgenic (NTG) littermates, were included in the present study. All mice were 12 to 14 weeks of age and 25 to 35 g of body weight. Both transgenic and non-transgenic mice were randomized to two groups, a saline group and an Iso/PE group. The Iso/PE group was treated with Iso (Sigma, 30mg/kg/d) and PE (Sigma, 30mg/kg/d) in saline with 0.1% ascorbic acid, whereas the saline group was treated only with saline and 0.1% ascorbic acid. All solutions were continuously infused for 7 days via osmotic minipumps (CharlesRiver). The minipumps were implanted in the left flank of the mice under isoflurane (2% in air) anesthesia. After seven days, mice were anesthetized, and body weight (BW) was determined. Subsequently, hemodynamic measurements were made, animals were sacrificed, hearts were weighed and collected for histology and molecular analysis.

Hemodynamic measurements

All hemodynamic measurements were made via invasive catheterization. A pressure transducer catheter (Micro-Tip 1.4 French, Millar Instruments Inc., Houston, TX, USA) was inserted into the right common carotid artery and advanced into the aortic artery to measure the aortic blood pressure using a MPVS 400 processor at a sample rate of 1.000 Hz with Chart 5 (Millar Instruments Inc., Houston, TX, USA). The catheter was subsequently advanced into the left ventricle. After stabilization, maximal LV pressure, LV end-diastolic pressure, and heart rate were continuously recorded. The maximal rates of LV pressure increase and decrease (systolic dPdt and diastolic dPdt, respectively) were derived from a differential tracing of left intraventricular pressure. Analyses were performed offline using PVAN 3.6 software.

Histology

For immunohistochemical analysis, hearts were fixed overnight with 10% neutral-buffered formalin at 4° C. After fixation, samples were subj ected to a dehydration series, embedded in paraffin and cut into 4 µM sections. Masson trichrome staining was performed to analyse collagen deposition. To quantify fibrosis, whole ventricle slice pictures were photographed using a Hamamatsu microscope, and fibrotic area was determined with Aperio's ImageScope software. Areas of fibrosis were calculated as percentage of total area of the left ventricle.

Quantitative Real-Time PCR

Quantitative Real-Time PCR (RT-PCR) was carried as described previously [4]. All RT-PCR primer sequences are shown in supplemental Table 1. Results were normalized to the housekeeping gene GAPDH levels and are expressed in arbitrary units.

Statistical analysis

All data are expressed as mean±SEM. The differences between two groups were determined by a Student's *t* test. Comparison between more than two groups data was assessed by One-Way ANOVA followed by post hoc Tukey test. A value of $P<0.05$ was considered to be statistically significant.

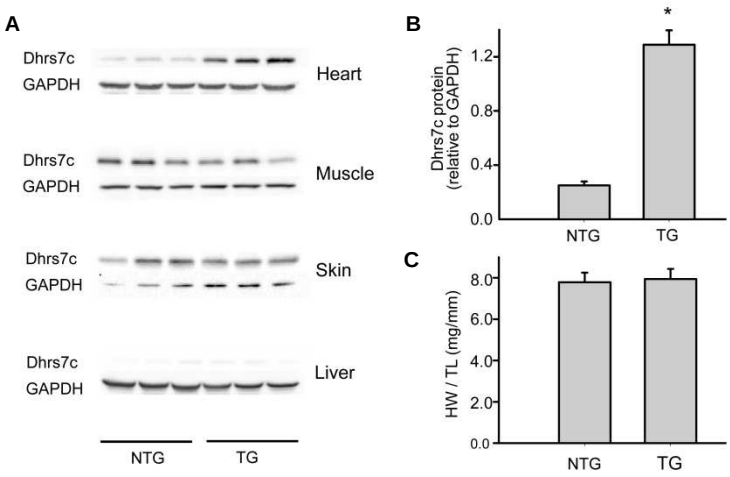


Figure 1. Baseline characteristics of Dhrs7c-transgenic mice

A, Western Blot of Dhrs7c in different tissues of TG and NTG animals. The overexpression of Dhrs7c in TG animals was limited to the heart. B, TG mice showed about 5 fold overexpression of Dhrs7c protein as compare with NTG littermates (*$P<0.05$, N=3). C, The ratio of heart weight (HW) to tibia length (TL) was similar in NTG and TG mice, (N=5).

Results

Baseline characterization of Dhrs7c transgenic mice

To examine the role of Dhrs7c *in vivo*, transgenic mice were generated with cardiac specific Dhrs7c overexpression, using an α-MHC promoter driven construct. A total of 5 transgenic founders were generated, but only two founder lines showed increased expression in the heart. The line with highest cardiac expression was used in this study. As shown in Figure 1A, this transgenic mouse line showed about 5 fold increased Dhrs7c protein expression in the heart, as compared to non-transgenic littermates. In other tissues, including skeletal muscle, skin and liver, no increased expression was observed, confirming that overexpression was confined to the heart (Figure 1B). We also analysed the heart weights of these animals, but did not observe differences between non-transgenic (NTG) and transgenic (TG) mice (Figure 1C), also expression of ANP, a HF-marker [5], was normal (data not shown). No morphological abnormalities were observed in hearts from the TG mice.

Iso/PE infusion and hypertrophy

Since Iso/PE downregulates endogenous Dhrs7c both *in vitro* and *in vivo* [2] we decided to infuse Iso/PE in TG and NTG litermates using osmotic minipumps. The control groups obtained saline infusion. As shown in Figure 2A and 2B, Iso/PE infusion for 7 days resulted in substantial downregulation of endogenous DHRS7c mRNA levels in the NTG group, whereas in the TG group these levels slightly decreased, but albeit remained much higher as compared to the NTG group. Both in TG and NTG groups, Iso/PE resulted in marked cardiac hypertrophy, as determined by the increase in heart weight (HW) to tibia length (TL), but no differences were observed between these Iso/PE infused groups (Figure 2C). Also expression of the HF marker ANP was significantly increased in the Iso/PE treated TG and NTG groups (Figure 2D), suggesting that there are no major differences in HF development in both groups.

Hemodynamic parameters

After 7 days continuous infusion hemodynamic parameters in the Iso/PE and saline treated groups were determined by invasive catheterization. These hemodynamic parameters are presented in Table 1. Albeit not all significant, Iso/PE infusion resulted in a trend towards higher heart rate, mean arterial pressure (MAP) and end systolic pressure (ESP) in both NTG and TG groups. No major differences in these parameters were observed between the TG and NTG groups. The contractile parameters (dPdtmax, dPdtmin, Tau g) appeared to be

slightly better in the TG group as compared to the NTG group, both after saline and Iso/PE infusion. Although none of them were significant, several p values almost reached significance (Tau g/HR p=0.059 and dPdtmax p=0.052 for TG versus NTG saline infusion and dPdtmin p=0.057 for TG versus NTG Iso/PE infusion) and a trend was visible (Table 1).

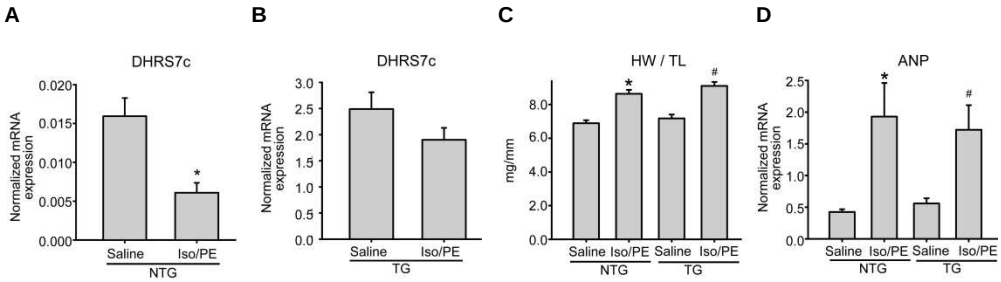


Figure 2. Effects of Iso/PE infusion on Dhrs7c mRNA expression and cardiac hypertrophy
A and B, Dhrs7c mRNA expression (corrected for GAPDH) was decreased in both NTG and TG mice after Iso/PE infusion, but only in NTG this downregulation was significant. Note the much higher expression levels in TG mice. C. Iso/PE infusion generated cardiac hypertrophy in both NTG and TG mice as shown by the ratio of heart weight to tibia length. D. ANP mRNA expression (corrected for GAPDH) was increased in both Iso/PE treated groups. N=9 for each group; *P<0.05 vs. NTG saline infusion, # P<0.05 vs. TG saline infusion.

Table 1. Hemodynamic parameters after saline and Iso/PE infusion.

Paramerters	NTG saline N7	NTG Iso/PE N=8	TG saline N=8	TG Iso/PE N=8
HR (bpm)	538.8±24.9	624.8±20.6*	601.7±26.9	650.6±35.0
MAP (mmHg)	83.4±3.3	89.8±2.9	78.6±2.8	96.1±4.6$^{\#}$
ESP (mmHg)	106.3±2.6	110.4±2.9	104.4±4.4	114.6±3.4
EDP (mmHg)	7.5±1.2	7.8±2.4	5.4±1.5	4.6±2.0
dPdtmax/Pmax(LV) (s^{-1})	92.9±4.0	89.4±4.3	104.4±9.9	87.7±5.1
dPdtmin/(ESP-EDP) (s^{-1})	-100.4±5.9	-70.3±4.0*	-107.0±7.2	-83.8±7.3”
dPdtmax (mmHg/s)	9887.8±368.6	9991.0±408.3	11742.6±835.0^	11040.2±1298.6
dPdtmin (mmHg/s)	-9878.7±536.6	-7181.1±359.2*	-11250.5±780.0	-9967.5±1207.6$^{\%}$
Tau g(ms)/HR(per sec)	1.09±0.10	1.43±0.16	0.8.±0.12$^{\$}$	1.18±0.20

All values are mean±SEM.

* p<0.05 vs. NTG saline infusion; $^{\#}$ p<0.05 vs. TG saline infusion; ^{S} p=0.059 vs. NTG saline infusion;

^ P=0.052 vs. NTG saline infusion; $^{\%}$ P=0.057 vs. NTG Iso/PE infusion.

Fibrosis and histology

Changes in the extracellular matrix (ECM) may affect cardiac function [6, 7] and we therefore performed Masson staining to quantify ECM deposition. Moreover a number of ECM markers were analysed by RT-PCR. As shown in Figure 3A and 3B, the ECM deposition was similarly increased in both NTG and TG Iso/PE treated groups. This corresponds with a similar increase in collagen I and III mRNA expression in the Iso/PE groups (Figure 3C, 3D). Also the expression of the ECM remodelling enzymes tissue inhibitor of metalloproteinases I (TIMP1) and matrix metalloproteinase 2 (MMP2) were significantly increased in the Iso/PE

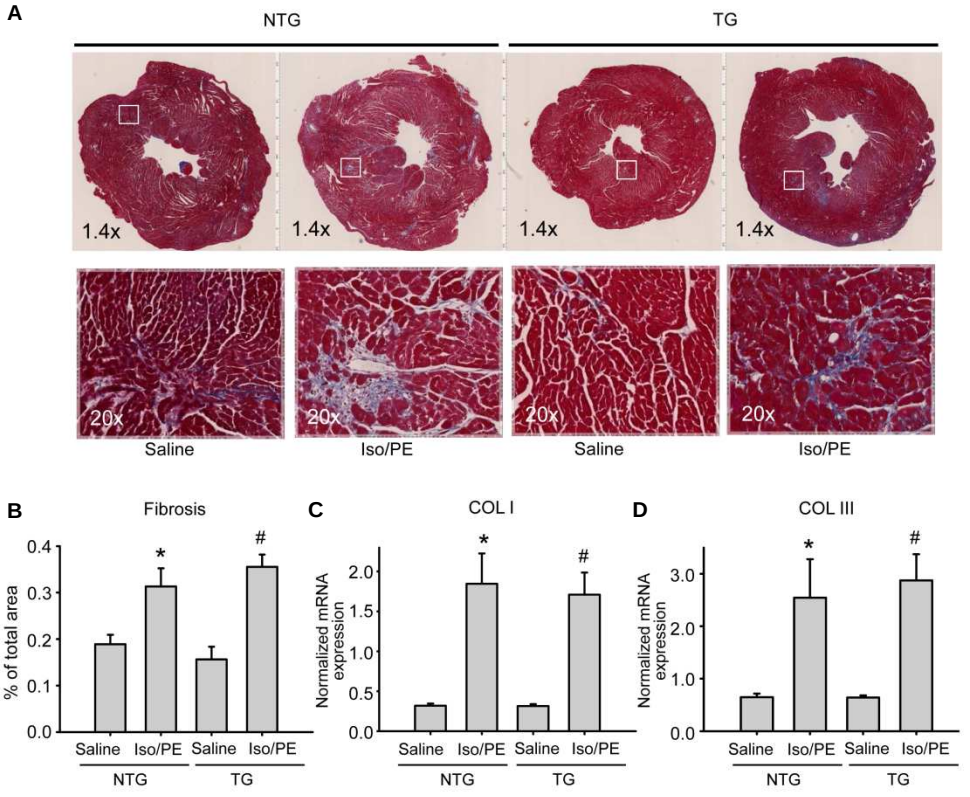


Figure 3. Collagen deposition and expression in NTG ad TG mice after 7 days saline and Iso/PE infusion.

A, Representative images of heart slices after Masson's trichrome staining. The collagen rich area stained blue. B, Quantification of the percentage fibrotic area over the total left ventricular area. C and D shows increased mRNA expression of Collagen I and Collagen III after Iso/PE infusion in both NTG and TG mice. N=9 for each group; *P<0.05 vs. NTG saline infusion, # P<0.05 vs. TG saline infusion.

groups and a similar trend was visible for TIMP2 (Figure 4). Interestingly, the increase in MMP2 expression in the TG treated group was significantly stronger than in the NTG Iso/PE treated group.

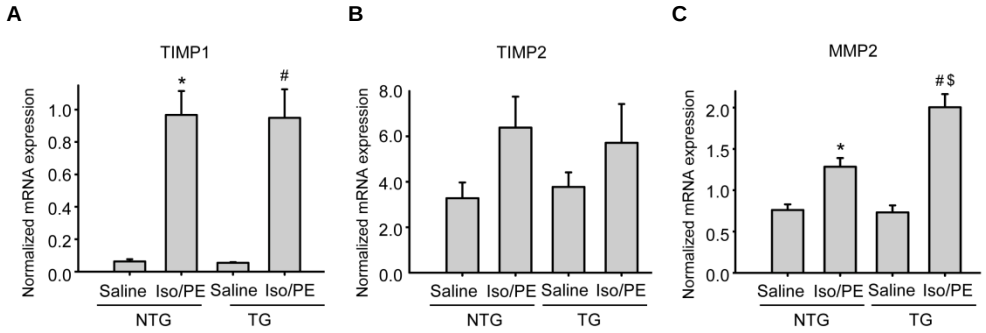


Figure 4. mRNA expression of ECM remodelling enzymes.
Expression was measured by RT-PCR and is relative to GAPDH. A, TIMP1. B, TIMP2. C, MMP2. N=9 for each group; *P<0.05 vs. NTG saline infusion, # P<0.05 vs. TG saline infusion, $ P<0.05 vs. NTG Iso/PE infusion.

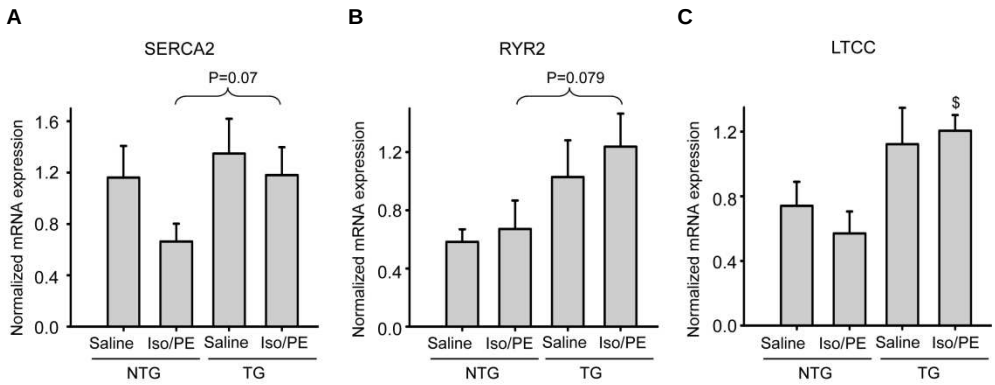


Figure 5. mRNA expression of calcium channel encoding genes
Expression was measured by RT-PCR and is relative to GAPDH. A, SERCA2. B, RYR2. C, LTCC. and B, SERCA2 and RYR2 mRNA expression was higher in the TG Iso/PE group as compared to the NTG Iso/PE group, though not statistically significant (P=0.007 and 0.079 respectively). $ P<0.05 vs. NTG Iso/PE infusion.

Calcium related gene expression

Dhrs7c is a sarcoplasmatic reticulum (SR) localized protein [2]. Since the SR plays an important role in controlling cellular calcium levels [8], we also investigated the mRNA expression of several calcium related SR proteins. In particular we determined the expression of the Ca^{2+} channelsryanodine receptor 2 (RYR2) [9], sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) [10]. In addition the expression of the T-tubule localized L-type Ca^{2+} channel (LTCC) [11] was investigated. SERCA2 was downregulated in the NTG group treated with Iso/PE, whereas SERCA2 remained almost constant in the TG group. RYR2 expression appeared to be somewhat elevated in the TG groups as compared to the NTG groups and the same was true for LTCC (Figure 5). The latter showed even a significant difference between the TG and NTG Iso/PE treated groups.

Discussion

Dhrs7c is a short chain dehydrogenase reductase, which is prominantly expressed in heart and is downregulated upon HF development and/or adrenergic stimulation. In the present study we investigated the effect of cardiac specific overexpression of Dhrs7c in transgenic mice. Despite a 5 fold overexpression of Dhrs7c protein, no spontaneous cardiac phenotype was observed. Also upon Iso/PE infusion, a condition that downregulates endogenous Dhrs7c expression and induces HF development, no major differences were observed. Only a slight trend towards improved function was observed in the TG groups and some small differences in expression of genes associated with fibrosis and Ca^{2+} handling could be observed.

To our knowledge this is the first study describing a transgenic mouse line with cardiac specific overexpression of Dhrs7c. Using this mouse line we aimed to dissect a potential cardiac phenotype associated with cardiac specific Dhrs7c overexpression. No spontaneous phenotype was observed in these transgenic mice and heart weights, expression of HF markers were all indistinguishable from the NTG group. This is in line with our previous *in vitro* studies using neonatal cardiomyocytes in which no obvious spontaneous phenotype was observed upon overexpression.

Previously we showed that in HF patients, in HF animal models and upon adrenergic stimulation Dhrs7c expression is profoundly downregulated. Moreover, overexpression of Dhrs7c in neonatal cardiomyocytes *in vitro* resulted only in cell death in the presence of adrenergic stimulation. We therefore decided to investigate cardiac function in Dhrs7c-TG mice after 7 days Iso/PE infusion. Surprisingly, no major phenotype was

observed. Only in parameters for contractility some differences, albeit just not significant, were observed between NTG and TG groups. In particular the relaxation constant (dPdtmin) and the contraction constant (dPdtmax) appeared to be improved in the TG group as compared to the NTG group and also a trend towards improved Tau g was observed in the TG group. This could be related to changes in the ECM [6, 7], but no obvious differences were observed in collagen expression or in fibrotic scores. Only, MMP2 expression was significantly elevated in Dhrs7c-TG mice treated with Iso/PE as compared to the NTG group. Whether this indicates that there might be more subtle changes in ECM function requires more detailed investigation of the ECM matrix. MMP2 has previously been shown to be a cAMP responsive gene [12] and hence the elevated upregulation of MMP2 may be caused by a hyper β-adrenergic response in the TG cardiomyocytes. It might therefore be interesting to investigate the expression of other cAMP responsive MMPs such as MT1-MMP and MMP13.

The observed small differences in cardiac function could also be mediated by altered calcium handling [13]. We therefore determined RYR2 [9], SERCA2 [10] and LTCC [11] mRNA expression. All the three genes were higher in TG than in NTG mice after Iso/PE infusion, though only LTCC was statistically significant. Whether this explains the observed small changes in contractility would require further research, but all these genes have been shown to be associated with alterations in Ca^{2+} handling and are linked to different cardiac diseases, such as cardiac myopathy, arrhythmias and heart failure [9, 11, 14, 15].

The lack of major cardiac differences in the Dhrs7c TG mice could have multiple reasons. First, Dhrs7c might only play a subtle role in the heart or functions into pathways that we did not investigate. The strong conservation of this gene in all vertebrates does, however, indicate an important function. The absence of a clear phenotype might therefore also be related to the limited overexpression (5 fold), which may not be sufficient to elicit a phenotype. *In vitro*, a much stronger phenotype was observed which could be due to the much stronger overexpression (>20 fold), but may also be related to the use of neonatal rat cardiomyocytes that express lower endogenous levels as compared to adult cardiomyocytes and might be more susceptible to Dhrs7c overexpression. We did not check the expression of other Dhrs7c family members (like Dhrs7 and Dhrs7b) and we cannot exclude that repression of these genes might compensate for Dhrs7c overexpression in the TG animals. Finally, we only analysed it in relatively young animals and it may be rewarding to evaluate whether overexpression of Dhrs7c would generate a stronger phenotype in aged animals.

In conclusion, we successfully generated a transgenic mouse with cardiac-specific Dhrs7c overexpression. The present study provides a basic characterization of this TG mouse line. The Dhrs7c-TG mice showed no spontaneous cardiac phenotype at baseline, or after Iso/PE infusion. However, some trends were visible, and several molecular markers did

show significant changes. Further investigations will be required to reveal the function of Dhrs7c in the heart.

Acknowledgements

We like to thank Bart van der Sluis for his help in generating the Dhrs7c transgenic mice. We like to thank Bianca Meijeringh for expert biotechnical assistance and Martin Dokter for performing the histology on cardiac tissue.

References

1. Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, Stromberg A, van Veldhuisen DJ, Atar D, Hoes AW, Keren A, Mebazaa A, Nieminen M, Priori SG, Swedberg K, ESC Committee for Practice Guidelines (CPG). ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: The task force for the diagnosis and treatment of acute and chronic heart failure 2008 of the european society of cardiology. developed in collaboration with the heart failure association of the ESC (HFA) and endorsed by the european society of intensive care medicine (ESICM). *Eur J Heart Fail*. 2008;10:933-989.

2. Lu B, Tigchelaar W, Ruifrok WP, van Gilst WH, de Boer RA, Sillje HHW. *DHRS7c*, a novel cardiomyocyte expressed gene that is downregulated by adrenergic stimulation and in heart failure. *Eur J Heart Fail*. In press.

3. Gulick J, Subramaniam A, Neumann J, Robbins J. Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J Biol Chem*. 1991;266:9180-9185.

4. Lu B, Mahmud H, Maass AH, Yu B, van Gilst WH, de Boer RA, Sillje HH. The Plk1 inhibitor BI 2536 temporarily arrests primary cardiac fibroblasts in mitosis and generates aneuploidy in vitro. *PLoS One*. 2010;5:e12963.

5. Yasue H, Obata K, Okumura K, Kurose M, Ogawa H, Matsuyama K, Jougasaki M, Saito Y, Nakao K, Imura H. Increased secretion of atrial natriuretic polypeptide from the left ventricle in patients with dilated cardiomyopathy. *J Clin Invest*. 1989;83:46-51.

6. Weber KT, Sun Y, Tyagi SC, Cleutjens JP. Collagen network of the myocardium: Function, structural remodeling and regulatory mechanisms. *J Mol Cell Cardiol*. 1994;26:279-292.

7. Diez J, Querejeta R, Lopez B, Gonzalez A, Larman M, Martinez Ubago JL. Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients. *Circulation*. 2002;105:2512-2517.

8. Priori SG, Chen SR. Inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis. *Circ Res*. 2011;108:871-883.

9. Meissner G. Molecular regulation of cardiac ryanodine receptor ion channel. *Cell Calcium*. 2004;35:621-628.

10. Kawase Y, Hajjar RJ. The cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase: A potent target for cardiovascular diseases. *Nat Clin Pract Cardiovasc Med*. 2008;5:554-565.

11. Satin J, Schroder EA, Crump SM. L-type calcium channel auto-regulation of transcription. *Cell Calcium*. 2011;49:306-313.

12. Guo D, Kassiri Z, Basu R, Chow FL, Kandalam V, Damilano F, Liang W, Izumo S, Hirsch E, Penninger JM, Backx PH, Oudit GY. Loss of PI3Kgamma enhances cAMP-dependent MMP remodeling of the myocardial N-cadherin adhesion complexes and extracellular matrix in response to early biomechanical stress. *Circ Res*. 2010;107:1275-1289.

13. Lompre AM, Hajjar RJ, Harding SE, Kranias EG, Lohse MJ, Marks AR. Ca2+ cycling and new therapeutic approaches for heart failure. *Circulation*. 2010;121:822-830.

14. Zhou Q, Xiao J, Jiang D, Wang R, Vembaiyan K, Wang A, Smith CD, Xie C, Chen W, Zhang J, Tian X, Jones PP, Zhong X, Guo A, Chen H, Zhang L, Zhu W, Yang D, Li X, Chen J, Gillis AM, Duff HJ, Cheng H, Feldman AM, Song LS, Fill M, Back TG, Chen SR. Carvedilol and its new analogs suppress arrhythmogenic store overload-induced Ca2+ release. *Nat Med*. 2011;17:1003-1009.

15. Stolen TO, Hoydal MA, Kemi OJ, Catalucci D, Ceci M, Aasum E, Larsen T, Rolim N, Condorelli G, Smith GL, Wisloff U. Interval training normalizes cardiomyocyte function, diastolic Ca2+ control, and SR Ca2+ release synchronicity in a mouse model of diabetic cardiomyopathy. *Circ Res*. 2009;105:527-536.

Supplemental materials

Table S1. Sequences of oligonucleotide primers used for quantitative real-time PCR

Genes	5'-3' Forward	5'-3' Reverse
DHRS7c	GGAGAACAGGCCAGATTGTG	CTCGGAGGCAGTCAAAGAAG
ANP	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC
GAPDH	CATCAAGAAGGTGGTGAAGC	ACCACCCTGTTGCTGTAG
Collagen 1a1	CTTCACCTACAGCACCCTTGTG	CTTGGTGGTTTTGTATTCGATGAC
Collagen III	TCGGAACTGCAGAGACCTAAA	CCCCAGTTTCCATGTTACAGA
α-MHC	GACAACTCCTCCCGCTTTGG	AAGATCACCCGGGACTTCTC
β-MHC	CGAGGCAAGCTCACATATAC	GTCACAATCATGCCGTGCTG
TIMP1	CTGCTCAGCAAAGAGCTTTC	CTCCAGTTTGCAAGGGATAG
TIMP2	GGTACCAGATGGGCTGTGAGTG	CTGTGACCCAGTCCATCCAGAG
MMP2	CCCTGATGTCCAGCAAGTAG	GGAGTCTGCGATGAGCTTAG
SERCA2	CTGCTGGCAGCATGTATATC	ACAAACGGCTCTACAAAGGC
RYR2	TTGGCAGCAGAAGGATTTGG	GCACAAAGGTGCAGATAGAC
LTCC	AGATCCAGCCATCTCCAAAGAG	CCTTTTGTCGCTTTAGACATTCC

ANP, atrial natriuretic peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; α/β-MHC, α/β-myosin heavy chain; TIMP1/2, tissue inhibitor of metalloproteinases 1/2; MMP2, matrix metalloproteinase 2; SERCA2, sarcoplasmic reticulum Ca^{2+}-ATPase; RYR2, ryanodine receptor 2; LTCC, L-type calcium channel.

Chapter 7

Summary, general discussion and future perspectives

Heart failure (HF) is a complex and progressive disorder and is defined as a condition in which the heart cannot supply sufficient blood and oxygen to keep up the body's demands. Several pathological processes are involved in the onset and progression of HF including cardiac remodeling [1] and re-entering of the fetal gene program [2]. The latter is a hallmark of HF development, but the role of this altered gene program in HF is still poorly understood. Some gene expression changes appear to be beneficial, whereas others, like SERCA2 [3, 4], are clearly detrimental, as described in **Chapter 2**. The aim of this thesis was to generate an inventory of hypertrophy associated gene expression changes. This would provide a more elaborate view of all the gene expression changes and could reveal new hypertrophy related genes that were hitherto not linked to this disease. These novel genes could be subject to further investigation and in this thesis we also described the further investigation of one of the identified genes, termed Dhrs7c.

In **Chapter 2**, we extensively reviewed two major components of cardiac remodeling, hypertrophy and fibrosis. In addition gene expression changes associated with these processes were described. In order to generate a more elaborate list of genes differentially expressed in HF and hypertrophy, it was decided to investigate expression changes in multiple models. The latter could provide a more robust list of genes with HF/hypertrophy associated expression, and exclude model specific alterations. Two *in vivo* rat models of HF were chosen as well as an *in vitro* model of cultured primary neonatal rat cardiomyocytes. The latter would also allow detection of gene expression changes specific to cardiomyocytes.

In **Chapter 3**, we describe the set-up of the *in vitro* primary rat cardiomyocyte culturing system and the induction of hypertrophy by neurohormonal factors. Since fibroblast contamination is a general problem in primary cardiomyocyte cultures we also investigated whether a novel anti-proliferative drug, BI 2536, targeting polo-like kinase 1 (Plk1) [5], could prevent fibroblast overgrowth in these cultures. We could show that BI 2536 had a profound effect on cardiac fibroblast proliferation *in vitro* and arrested these cells in mitosis with an IC50 of about 43 nM. Further analysis revealed that prolonged mitotic arrest resulted in cell death for about 40% of cardiac fibroblasts. The remaining cells showed an interphase morphology with mostly multi and micro nuclei. This indicates that a significant number of primary fibroblasts were able to escape BI 2536 induced mitotic arrest and apparently became aneuploid. No effects were observed on cardiomyocytes and hypertrophic response upon endothelin-1 and phenylephrine stimulation was normal in the presence of BI 2536. This indicates that BI 2536 had no adverse effects on terminally differentiated cells and still

allowed proliferation independent growth induction in these cells. Thus, although BI 2536 could reduce fibroblast numbers, without apparent adverse effects to cardiomyocytes, it could not fully eliminate these cells from the cardiomyocyte cultures. Short culturing times to prevent overgrowth remain therefore a prerequisite in cardiomyocyte culturing.

Having established an *in vitro* cardiomyocyte culturing system to investigate hypertrophy, we subsequently started the investigation of differential gene expression in response to hypertrophic stimuli (**Chapter 4**). For this, cells were stimulated with isoproterenol (Iso, a β-adrenergic agonist), phenylephrine (PE, an α-adrenergic agonist) or endothelin-1 (ET-1). All these agents generated hypertrophy and induction of the fetal gene program, as determined by ANP expression. Subsequently, gene array analysis was performed for each culture condition. In parallel, gene expression was analysed in two different *in vivo* rat HF models, namely post-MI HF and Ren 2 rats that overexpress mouse renin thereby generating hypertension which results in HF development. Gene expression changes as compared to the respective controls (non-treated cardiomyocytes or non-HF rats) were determined and the obtained lists with differential expressions were compared with each other. This generated a concise list of 62 genes differentially expressed in hypertrophy/HF. Additional gene ontology analysis revealed clear differences between *in vitro* and *in vivo* models. The extracellular matrix, wounding and stress specific genes were enriched in the *in vivo* list; most of them are predominantly expressed by non-cardiomyocytes in heart tissue which explains the absence in the *in vitro* cardiomyocyte experiments. The terpenoid and steroid biosynthesis pathways on the other hand were upregulated in the *in vitro* models. Importantly, we identified the common known HF markers like ANP, BNP, TIMP1, FHL1 and LGALS3 validating this method [6-8]. Moreover, the presence of genes not previously associated to hypertrophy/HF suggests that this combined *in vitro*/*in vivo* approach is a powerful method to identify new genes potentially associated with HF. Amongst these are PTGIS, AKIP1 and Dhrs7c, which could be interesting targets for further investigations. The presence of TNFRSF12a in our list, which was identified as a HF indicator by others [9] during the course of our experiments, further confirmed that potential new HF associated genes could be identified by this method. Taken together, this study provided insight in the differences between the *in vivo* and *in vitro* models; and at the same time, it provided a robust set of hypertrophy/HF associated genes.

From the set of genes identified in **Chapter 4**, a short chain dehydrogenase/reductase, DHRS7c, demonstrated consistent decrease in all our models except in ET-1 treated

cardiomyocytes. In **Chapter 5**, we confirmed that DHRS7c was downregulated in our different animal HF models, not only at mRNA level, but also at protein level. Moreover, like in vitro, the expression of Dhrs7c could be downregulated in vivo by an Iso/PE infusion, suggesting that adrenergic stimulation could be an important mediator of this downregulation. The absence of ET-1 mediated downregulation in vitro, despite hypertrophy formation, did indicate that downregulation was not directly linked to hypertrophy, but instead might be correlated to adrenergic stimulation. Importantly, we could extend our investigations to human cardiac biopsies from HF patients and show that Dhrs7c is also downregulated in human HF patients. Therefore this could also have clinical relevance. Interestingly, Dhrs7c expression is confined to muscle, heart and skin indicating that it has a tissue specific function. Within the heart, expression was only observed within cardiomyocytes and was absent in the non-cardiomyocyte cell population. Dhrs7c appeared to localize to the endo/sarcoplasmatic reticulum in these cells, but so far we could not reveal its function in this organelle. Overexpression and silencing of Dhrs7c did not provide an obvious phenotype in vitro, except in the presence of adrenergic stimulation. Under these conditions, cell rapidly died, but the molecular mechanism behind this cell death remained unclear and did not appear to be apoptosis related. It did, however, suggest that downregulation could be important under conditions of adrenergic stimulation.

To further investigate the functional effects of Dhrs7c overexpression, a transgenic mouse with cardiac specific overexpression of Dhrs7c was generated, as described in **Chapter 6**. Out of 5 mouse lines only one transgenic (TG) mouse line showed substantial Dhrs7c overexpression of approximately 5 fold at the protein level. These TG mice behaved normally and did not show any obvious phenotype. The HF markers (e.g. ANP), fibrotic and hemodynamic parameters were also similar in TG and non-transgenic littermate (NTG) group after continuous adrenergic activation via Iso/PE infusion. Only some differences were observed in the expression of metalloproteinase II (MMP2) and L-type calcium channel (LTCC) in the TG Iso/PE group as compared to the NTG Iso/PE group. These differences are, however, minor and it is not clear if these differences are relevant. The lack of a spontaneous and Iso/PE induced phenotype might have numerous reasons as discussed in **Chapter 6**. One of them is the limited overexpression of only 5 fold, which might not be sufficient to elicit a phenotype. The strong conservation of this gene in vertebrates and its tissue specific expression clearly suggest an important evolutionary conserved function of this protein. Elucidation of its function would require biochemical analysis of this protein, including the identification of its potential substrates. We did try to purify sufficient recombinant protein to

perform such analysis, but were unfortunately not able to obtain it in a functional soluble form. In addition to biochemical analysis, the generation of a Dhrs7c knock-out mouse may shed a better light on the function of this intriguing protein.

Overall, in this thesis we (1) provided a method to inhibit fibroblast proliferation in cardiomyocyte culturing; (2) compared the gene expression between in vivo and in vitro models; (3) further investigated Dhrs7c, one of the novel HF associated genes from our gene list. Future studies on Dhrs7c would probably require the generation of a knock-out mouse to reveal its function. The generated gene list also contained a number of other interesting candidates and it may be rewarding to investigate other top-hits from our list, like AKIP1 and PTGIS.

References

1. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. experimental observations and clinical implications. Circulation. 1990;81:1161-1172.

2. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: A strong hypothesis. Heart Fail Rev. 2007;12:331-343.

3. Meyer M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. Circulation. 1995;92:778-784.

4. Schwinger RH, Bohm M, Schmidt U, Karczewski P, Bavendiek U, Flesch M, Krause EG, Erdmann E. Unchanged protein levels of SERCA II and phospholamban but reduced Ca2+ uptake and ca(2+)-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. Circulation. 1995;92:3220-3228.

5. Barr FA, Sillje HH, Nigg EA. Polo-like kinases and the orchestration of cell division. Nat Rev Mol Cell Biol. 2004;5:429-440.

6. Zhang QJ, Chen HZ, Wang L, Liu DP, Hill JA, Liu ZP. The histone trimethyllysine demethylase JMJD2A promotes cardiac hypertrophy in response to hypertrophic stimuli in mice. J Clin Invest. 2011;121:2447-2456.

7. de Boer RA, Yu L, van Veldhuisen DJ. Galectin-3 in cardiac remodeling and heart failure. Curr Heart Fail Rep. 2010;7:1-8.

8. Kramer F, Milting H. Novel biomarkers in human terminal heart failure and under mechanical circulatory support. Biomarkers. 2011;16 Suppl 1:S31-41.

9. Chorianopoulos E, Heger T, Lutz M, Frank D, Bea F, Katus HA, Frey N. FGF-inducible 14-kDa protein (Fn14) is regulated via the RhoA/ROCK kinase pathway in cardiomyocytes and mediates nuclear factor-kappaB activation by TWEAK. Basic Res Cardiol. 2010;105:301-313.

Nederlandse samenvatting

Hartfalen is een complexe en progressieve aandoening waarbij het hart niet genoeg bloed en zuurstof kan rondpompen om aan de behoefte van het lichaam te voldoen. Verschillende pathologische processen zijn betrokken bij het ontstaan en het verdere verloop van hartfalen, waaronder hart remodeling [1]. Dit omvat onder anderen hypertrofie (groei) van de cardiomyocyten (hartspiercellen), fibrose, en de reactivatie van foetale genexpressie [2]. Deze veranderde genexpressie is een belangrijk kenmerk van hartfalen, maar de rol hiervan is nog niet duidelijk. Terwijl sommige veranderingen in de genexpressie een gunstig effect lijken te hebben, hebben andere, zoals SERCA2 [3, 4], een duidelijk negatief effect, zoals beschreven in **hoofdstuk 2**. Het doel van het in dit proefschrift beschreven onderzoek, was het maken van een overzicht van alle genexpressie veranderingen die optreden tijdens hypertrofie en hartfalen ontwikkeling. Een dergelijk overzicht zou een beter inzicht kunnen geven in hartfalen en zou kunnen leiden tot de identificatie van genen die nog niet eerder met hartfalen in verband zijn gebracht. Deze nieuwe genen kunnen vervolgens nader onderzocht worden, zoals dit in dit proefschrift is gedaan voor één van de nieuw geïdentificeerde genen, genaamd Dhrs7c.

In **hoofdstuk 2** worden de twee belangrijkste componenten van hart remodeling beschreven, namelijk hypertrofie en fibrose. Ook de bekende genexpressie veranderingen van de genen die betrokken zijn bij deze processen worden hier beschreven. Om een uitgebreide lijst te verkrijgen van alle genen, die differentieel tot expressie komen in zowel hartfalen als hypertrofie hebben we vervolgens besloten de genexpressie veranderingen in diverse modellen van hartfalen/hypertrofie te bestuderen. Dit zou een betrouwbare lijst kunnen opleveren met genen waarvan de expressie geassocieerd is met hartfalen/hypertrofie en ook model specifieke veranderingen aan het licht kunnen brengen. Er werden hiervoor twee *in vivo* rat modellen van hartfalen gebruikt, gecombineerd met een *in vitro* model bestaande uit een cel kweek van primaire neonatale rat cardiomyocyten. Door het gebruik van dit *in vitro* model kon ook de cardiomyocyt specifieke genexpressie bestudeerd worden.

In **hoofdstuk 3** hebben we de opzet beschreven van het *in vitro* kweek systeem van primaire neonatale rat cardiomyocyten en de inductie van hypertrofie door neurohumorale factoren hierin. De contaminatie van primaire cardiomocyten met fibroblasten is in deze kweek een algemeen bekend probleem. Daarom hebben we eerst onderzocht of een nieuw anti-proliferatieve stof, BI 2536, dat als target polo-like kinase 1 (Plk1) [5] heeft, de vermeerdering van fibroblasten kon voorkomen. We hebben laten zien dat BI 2536 een remmend effect had op de proliferatie van de fibroblasten *in vitro* en dat de celcyclus van deze cellen stopte in

mitose met een IC50 van ongeveer 43 nM. Verdere analyse liet zien dat gedurende een langere stagnatie van de celcyclus ongeveer 40% van de cardiale fibroblasten dood gingen. De overblijvende cellen hadden de morfologie van interfase cellen met meestal meerdere kernen en microkernen. Dit wijst erop dat een significant aantal primaire fibroblasten konden ontkomen aan de door BI 2536 geïnduceerde stagnatie in de mitotische fase, en blijkbaar aneuploïd werden. Er werden geen effecten gezien op de cardiomyocyten en de hypertrofe respons gedurende stimulatie met endotheline-1 en phenylephrine was normaal na de behandeling met BI 2536. Dit duid erop dat BI 2536 geen bijwerkingen had op terminaal gedifferentieerde cellen en dat proliferatie onafhankelijke groei-inductie nog steeds mogelijk was. Kortom, hoewel BI 2536 het aantal fibroblasten kon verminderen zonder bijwerkingen op cardiomyocyten te hebben, kon BI 2536 niet alle fibroblasten uit de cardiomyocyten kweek elimineren.

Nadat we de methode voor de *in vitro* cardiomyocyten kweek hadden opgezet om hypertrofie te onderzoeken, hebben we de verschillen in genexpressie in reactie op hypertrofe stimuli onderzocht (**hoofdstuk 4**). Hiervoor werden de cellen gestimuleerd met isoproterenol (Iso, een β-adrenerge antagonist), phenylephrine (PE, een α-adrenerge agonist) of endotheline-1 (ET-1). Al deze stoffen veroorzaakten hypertrofie en de reactivatie van de expressie van de foetale genen, welke we hebben bepaald door de ANP expressie te meten. Vervolgens hebben we een gene array analyse gedaan op elke kweek conditie en op twee verschillende *in vivo* modellen van hartfalen bij de rat, namelijk post-myocard infarct (post-MI) en in Ren 2 ratten. Laatstgenoemde ratten brengen het muizen renine gen tot overexpressie waardoor de dieren een hoge bloeddruk krijgen dat uiteindelijk leidt tot de ontwikkeling van hartfalen. De veranderingen in de genexpressie ten opzichte van de bijbehorende controles (niet behandelde cardiomyocyten of ratten zonder hartfalen) werden bepaald en de verschillen in de expressies werden met elkaar vergeleken. Dit resulteerde in een beknopte lijst met 62 genen die verschillend tot expressie kwamen in de hypertrofie/hartfalen modellen. Gene ontology analyse liet tevens duidelijke verschillen zien tussen de *in vitro* en *in vivo* modellen. Extracellulaire matrix genen en genen die geactiveerd worden bij verwonding en stress waren veel voorkomend in de lijst met de *in vivo* modellen; de meesten komen voornamelijk tot expressie in de niet-cardiomyocyt cellen in het hart wat tevens de afwezigheid verklaarde in de *in vitro* cardiomyocyten experimenten. Daar tegenover staat dat de terpenoïde en steroïde biosynthese reacties verhoogd waren in de *in vitro* modellen. Als positieve bevestiging van de analyse hebben we de algemeen bekende markers voor hartfalen gevonden zoals ANP, BNP, TIMP1, FHL1 en LGALS3 [6-8]. Dat we naast deze bekende genen

ook genen vonden die niet eerder geassocieerd werden met hypertrofie/hartfalen maakt duidelijk dat deze combinatie van *in vitro/in vivo* modellen een goede benadering is om nieuwe genen te identificeren, die mogelijk betrokken zijn bij hartfalen. Enkele van deze nieuwe genen zijn PTGIS, AKIP1 en Dhrs7c welke interessant zijn voor verder onderzoek. De aanwezigheid van TNFRSF12a in onze lijst, welke tijdens onze studie ook door anderen werd geïdentificeerd als een hartfalengeassocieerd [9] gen bevestigde dat nieuwe hartfalen genen geïdentificeerd konden worden met deze methode. Kortom, deze studie gaf inzichten in de verschillen tussen *in vivo* en *in vitro* modellen en leverde tegelijkertijd een beknopte lijst van hypertrofie/hartfalen geassocieerde genen op.

Van de geïdentificeerde genen die werden beschreven in **hoofdstuk 4**, liet een short chain dehydrogenase/reductase, DHRS7c, een consistente verlaging in de expressie zien in al onze modellen met uitzondering van ET-1 behandelde cardiomyocyten. In **hoofdstuk 5** hebben we bevestigd dat de expressie van DHRS7c naar beneden ging in onze verschillende diermodellen van hartfalen, niet alleen op mRNA niveau maar ook op eiwit niveau. Net als in de *in vitro* modellen was de expressie van Dhrs7c verlaagd na iso/PE infusie *in vivo*. Dit suggereerde dat adrenerge stimulatie belangrijk zou kunnen zijn voor deze verlaging in de genexpressie. Dat ET-1 geen verlaging van de genexpressie *in vitro* veroorzaakte, ondanks de hypertrofie ontwikkeling, laat zien dat verminderde expressie van Dhrs7c niet direct gecorreleerd is met hypertrofie maar waarschijnlijk gerelateerd is aan adrenerge stimulatie. Daarnaast konden we ook laten zien dat de expressie van Dhrs7c verminderd was in patiënten met hartfalen door humane cardiale biopten te analyseren. Dit effect kan dus mogelijk klinische relevantie hebben. Dhrs7c wordt alleen in de spier, het hart en de huid tot expressie gebracht, wat kan duiden op een weefsel specifieke functie. In het hart werd de expressie alleen waargenomen in de cardiomyocyten en niet in de niet-cardiomyocyt cellen. In deze cellen leek Dhrs7c gelokaliseerd te zijn in het endo/sarcoplasmatische reticulum, maar tot nog toe hebben we niet de functie in dit organel kunnen ontrafelen. We hebben ook geen duidelijk fenotype waargenomen in cellen waarin de expressie van Dhrs7c *in vitro* verhoogd (overexpressie) of verlaagd (silencing) werd, behalve in de aanwezigheid van adrenerge stimulatie. Onder deze condities gingen de cellen snel dood, maar de moleculaire mechanismen achter deze celdood bleven onduidelijk. Het leek echter niet apoptose gerelateerd te zijn. Deze waarneming duidde er op dat verminderde expressie van Dhrs7c belangrijk zou kunnen zijn tijdens adrenerge stimulatie.

Om de functionele effecten van de overexpressie van Dhrs7c te onderzoeken hebben we een transgene (TG) muis gemaakt met hart specifieke overexpressie van Dhrs7c. Dit hebben we beschreven in **hoofdstuk 6**. Van de vijf verschillende foklijnen gaf slechts één TG muislijn een substantiële vijfvoudige Dhrs7c verhoging op eiwit niveau. Deze dieren lieten geen afwijkend gedrag zijn en hadden geen spontaan fenotype. De hartfalen markers (bijvoorbeeld ANP), maar ook de fibrotische en hemodynamische parameters waren gelijk in de TG en niet transgene dieren (NTG), ook na een continue adrenerge activatie via iso/PE infusie. Alleen in de expressie van metalloproteinase II (MMP2) en L-type Calcium channel (LTCC) werden enkele verschillen waargenomen tussen de TG Iso/PE groep en de NTG Iso/PE groep. Deze verschillen waren echter klein en het is nog niet duidelijk of deze verschillen relevant zijn. Het ontbreken van een spontaan en Iso/PE geïnduceerd fenotype kan meerdere redenen hebben die besproken worden in **hoofdstuk 6**. Een van deze redenen is de beperkte overexpressie van slechts 5-voud, wat misschien niet genoeg is om een fenotype te induceren. De sterke conservatie van dit gen in gewervelden en de tissue specifieke expressie suggereren echter duidelijk een belangrijke evolutionair geconserveerde functie van dit eiwit. Om de functie te verhelderen zijn biochemische analyses van dit eiwit nodig, waaronder de identificatie van de potentiële substraten. We hebben geprobeerd om genoeg recombinant eiwit te maken om deze analyses te doen, maar we hebben helaas dit eiwit niet in oplosbare vorm kunnen zuiveren. Naast de biochemische analyses zou de Dhrs7c knock-out muis ook kunnen helpen de functie van dit fascinerende eiwit op te helderen.

Kortom, in dit poefschrift hebben we (1) een methode beschreven om de proliferatie van fibroblasten te remmen in de kweek van cardiomyocyten; (2) de genexpressies vergeleken tussen *in vivo* en *in vitro* modellen van hartfalen; (3) de expressie en mogelijke functie van Dhrs7c, een nieuw ontdekt gen dat niet eerder met hartfalen was geassocieerd, onderzocht. In toekomstig onderzoek, zal waarschijnlijk een zogenaamde knock-out muis moeten worden gemaakt, waarmee de functie van Dhrs7c opgehelderd kan worden. De verkregen lijst met genen omvatte ook een aantal andere interessante kandidaten en verder onderzoek van genen, zoals AKIP1 en PTGIS, zou zeer lonend kunnen zijn.

References

1. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. experimental observations and clinical implications. Circulation. 1990;81:1161-1172.

2. Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: A strong hypothesis. Heart Fail Rev. 2007;12:331-343.

3. Meyer M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. Circulation. 1995;92:778-784.

4. Schwinger RH, Bohm M, Schmidt U, Karczewski P, Bavendiek U, Flesch M, Krause EG, Erdmann E. Unchanged protein levels of SERCA II and phospholamban but reduced Ca2+ uptake and ca(2+)-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. Circulation. 1995;92:3220-3228.

5. Barr FA, Sillje HH, Nigg EA. Polo-like kinases and the orchestration of cell division. Nat Rev Mol Cell Biol. 2004;5:429-440.

6. Zhang QJ, Chen HZ, Wang L, Liu DP, Hill JA, Liu ZP. The histone trimethyllysine demethylase JMJD2A promotes cardiac hypertrophy in response to hypertrophic stimuli in mice. J Clin Invest. 2011;121:2447-2456.

7. de Boer RA, Yu L, van Veldhuisen DJ. Galectin-3 in cardiac remodeling and heart failure. Curr Heart Fail Rep. 2010;7:1-8.

8. Kramer F, Milting H. Novel biomarkers in human terminal heart failure and under mechanical circulatory support. Biomarkers. 2011;16 Suppl 1:S31-41.

9. Chorianopoulos E, Heger T, Lutz M, Frank D, Bea F, Katus HA, Frey N. FGF-inducible 14-kDa protein (Fn14) is regulated via the RhoA/ROCK kinase pathway in cardiomyocytes and mediates nuclear factor-kappaB activation by TWEAK. Basic Res Cardiol. 2010;105:301-313.

Acknowledgements

Time flies by. It seems just yesterday that I arrived in this mysterious country and started a totally new, but interesting life. I still remember four and a half years ago, in the chilly midnight of 16 August 2007 when Cheng waited for me on the platform at the railway station of Groningen. Later at dawn of the next day, Dr. Qu Ning, whom I had never met before, drove me to Cheng and Lili's. If I was a fatalist, I would have known the PhD life here would not be easy, but with the help of many warm-hearted people, this task would not be unachievable either. And now, in the last part of my thesis, I would like to thank all the people who have walked along beside me and have helped me during these years.

First of all, I want to express my gratitude to my promoter, Prof. dr. Wiek H. van Gilst. Dear Wiek, thank you for your support in my scientific research during these four years. You always have a broad view of the design of the experiments and the translation of the results. You let me understand that in research, as in life, it is important to face the reality and to focus on the important things. And I have no doubt that the group of Experimental Cardiology will become larger and stronger in the future.

Secondly, my utmost thanks go to my daily supervisor, Dr. Herman H. W. Silljé. Dear Herman, your passion in research and your enthusiasm in learning have had a great impact on me. Without your help in my studies, I cannot imagine I would have ever made it this far with my PhD in the Netherlands. Without your correction of the manuscripts, I would have never finished this thesis. I will always appreciate your patient guidance in my research work. And I will always remember you being there any time when there is problem to be solved. I hope someday I have the ability and opportunity to return your kindness and support when I am well-established in China. Again, thank you!
I would also like to thank my other co-promoter, Dr. Rudolf A. de Boer. Dear Rudolf, I am impressed by your efficiency and optimism on research work. You always find the brighter side even in a disappointing situation. I valued the great combination of yours and Herman's opinion which really speeded up my project. I have learned a lot from you, especially since I am going back to China to be a clinical doctor, and also continue my research interests.

My word of thank goes also to the members of my reading committee, Prof. dr. M.P. van den Berg, Prof. dr. A.A. Voors and Prof. dr. J.H. Kingma, for critically evaluating my thesis.

A special thanks to Riekje Banus, Mathilde Pekelaar and Maaike H. Bansema from GUIDE, for all the support and useful information provided for PhD students.

I owe my sincere thanks to the people from Experimental Cardiology. Thanks to all PhD students and post docs: Reinout, Irma, Hisko, Liza, Anne-Margreet, Mariusz, Willem-Peter, Daan, Megan, Niek, Vincent, Atze, Leonie, Jardy, Lennaert, Michael, Frank, Nicolas, Hasan, Irene, and Maxi, for all the wonderful presentations and good ideas you gave at every Lunch and PhD meeting. I hope we can work together again in the future. Dear Reinout, I could always turn to you for help when I had a problem with the Millar data. Dear Irma, thank you for the detailed information on preparing for thesis printing and promotion. Dear Megan, it is so nice to have a native-English speaker next to me and help me with the grammar.
I would like to thank Carla, Danielle and Marjan. Thank you for your help.
I am also grateful for the technical support of Martin, Linda, Silke, Bibiche, Inge, Janny, Annet and Germaine. Dear Martin, thank you for your efficient work in immunochemistry staining. Dear Silke, you are always willing to help people. It is very nice to have you in the group. The people from CDL, Bianca, Annemiek, Andre and Michel, thank you for helping me with the animal experiment. Thanks to Klaas from the Imaging Center.
I would like to thank Maggie, Peter, Adriaan and Marry at Clinical Pharmacology. Dear Adriaan, thank you for sending me to central station before I leave for China. You were the guide for my badminton skills. I hope someday I can be your tour guide in Harbin when you travel around China.

My warmest thanks go to Wardit and Zhang Deli, thank you for being my paranimfen. I know you did a lot for me, especially when I was in China. Thank you for everything.

Living far away from home is not always easy. I was lucky to have so many Chinese friends here in Groningen accompany me through the happy and unhappy days. Dear Niu Xiaoyu, I enjoyed all the talks we had, and appreciated your wise words very much. You are not only a co-jogger but a friend indeed. Dear Qin Si, I wish everything goes well for you in the United States. Dear Zhang Chunling, how about your work in Shanhai? Good luck with your new life. Dear Liu Gaifen, thank you for your suggestions concerning my study and my life. I am looking forward to meeting you in Beijing in the future. Dear Qin Li, you are a good sport mate, and you are more than that. I wish you all the best. Dear Lu Wenli, I know you will have your defense on January, and you will travel around Europe with your husband after that. I wish you success and enjoy your trip. Dear Lia, Jin Xin, Lang Shuting, Sun Xiaoxiao and Camie, I enjoyed our corridor- and kitchen-talk very much. We are from different areas and countries, and I learned a lot from all of you. Hope our friendship will last forever.

Of course, many thanks to the HMUers: Yu Lili & Qian Cheng, Han Lina & Zhao Yan, Tan Hongtao, Wei Yunwei, Yu Hongjuan and Yin Meimei. Dear Cheng and Lili, I really appreciated your help at the beginning of my time in Groningen. Dear Hongtao and Yunwei, I hope you have your promotion soon. Dear Lina and Yan, your daughter looks so lovely, best wishes to her. Dear Hongjuan, I am amazed by your hard work, good luck to you.

Dear Li Jiang, the 80 km-one-day cycling was really tough, and unforgettable! Thanks to Liu Bo, Liu Qi, Liang Zheng, Bu Shaochong, Cai Yingdan, Jiang Yi, Liu Yuxuan, Chen Bing and Li Kun. I cherish all the good times we have had in Groningen. Keep in touch and hope to see you in Harbin one day.

I would like to thank Fu Jingyuan, Ding Ning, Yu Zilin, Zhang Faya & Li Peiqin, Wu Bian & Zhou Qiyun, Lee Ee Soo, Ye Qingsong, He Yan, Xue Ruiqi, Liu Xiaomei, Lu Mingtao, Du Xiaoguang, Mu Dongdong and Wang Yi. Dear Ning and Zilin, thank you for all your effort to unite the students in Groningen. Dear Ee Soo, I will remember that if I want to chat with old couples during a cruise, always start with the question, “How many times have you been on a cruise?” And, “which is the most unforgettable one?” Dear Jingyuan, you are always kind to me, thank you.

I also want to express my gratitude to my supervisor in China. Dear Prof. dr. Yu Bo, without your support I would have never achieved my degree in China. I am appreciative of your kindness and constant readiness to help me. Thank you.

My special thanks to Zhai Aixia, Diao Liwei, Lu Libo, Liu Haixia, Wang Xiaodong, Li lili and Du Wenjuan. Thank you very much for your help.

Finally, never enough thanks to my beloved parents and my sister. Dear Mom and Dad, thank you for all the unconditional love you have given to me. I know I am not a perfect daughter, but in my eyes, you are always the best parents. My dear sister, thank you so much for your support! Dear Tianyu, I feel happy and at peace whenever I hear your sweet voice. I wish for you to grow up with happiness and joy.

致 谢

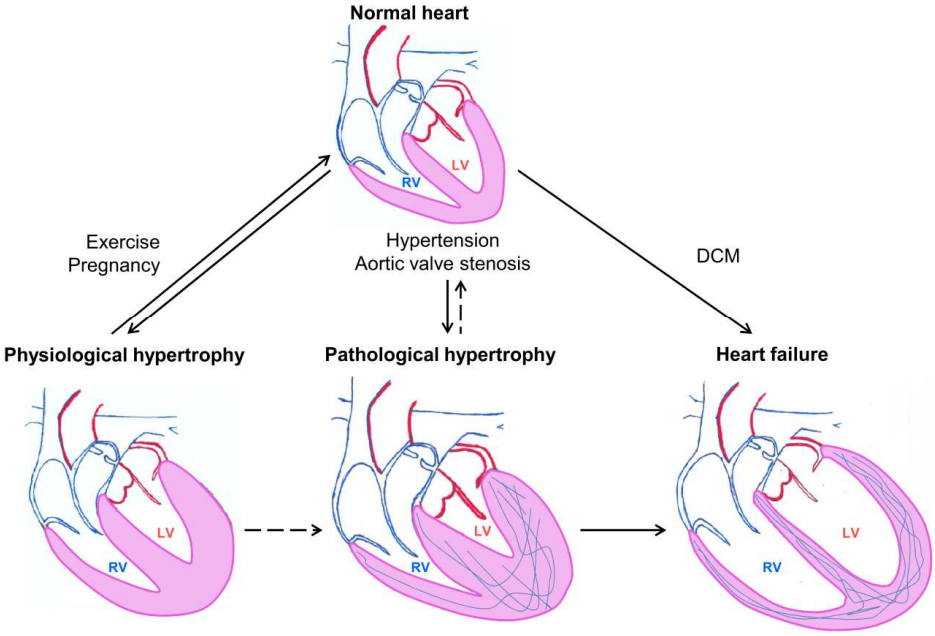
首先感谢我的导师于波教授多年来对我的指导、帮助。于老师严谨求实的治学态度和谦逊豁达的品格修养在潜移默化中熏陶、影响着我，使我受益终生。

感谢我的父母，感谢你们多年来的鼓励和支持，是你们给了我前进的信心和坚持的勇气，希望你们能够健康、长寿！感谢我的妹妹，你总是在我最需要的时候给我安慰、给我建议和帮助，谢谢你！非常感谢你几年来对父母的悉心照料，希望你幸福！感谢王天禹，谢谢你给全家人带来如此多的欢乐；希望你能早日读懂英文，把上一页的内容翻译给姥姥和姥爷听。

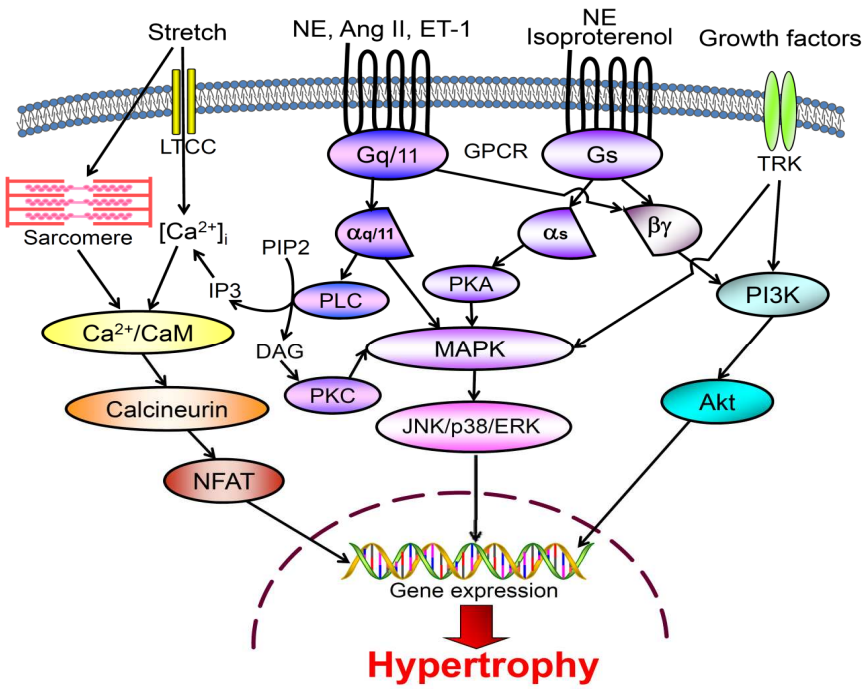
衷心感谢所有关心和帮助过我的人！

Coloured figures

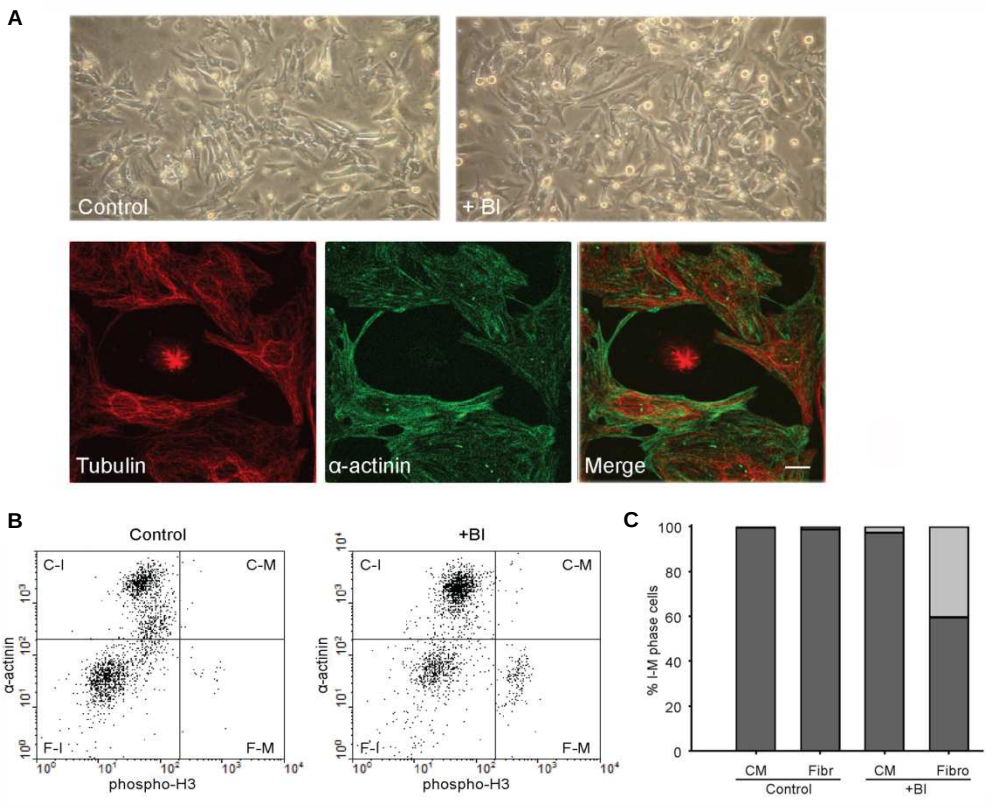
Chapter 1-Figure 1, page 11



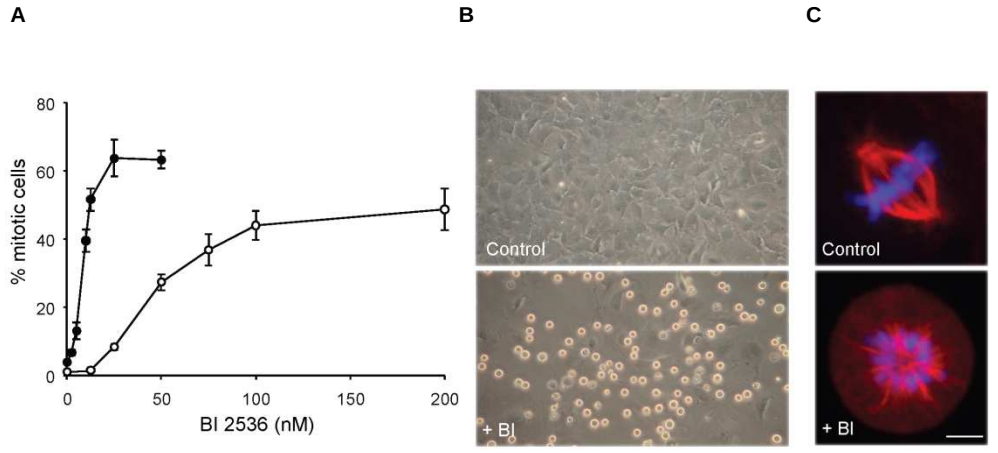
Chapter 2-Figure 1, page 20



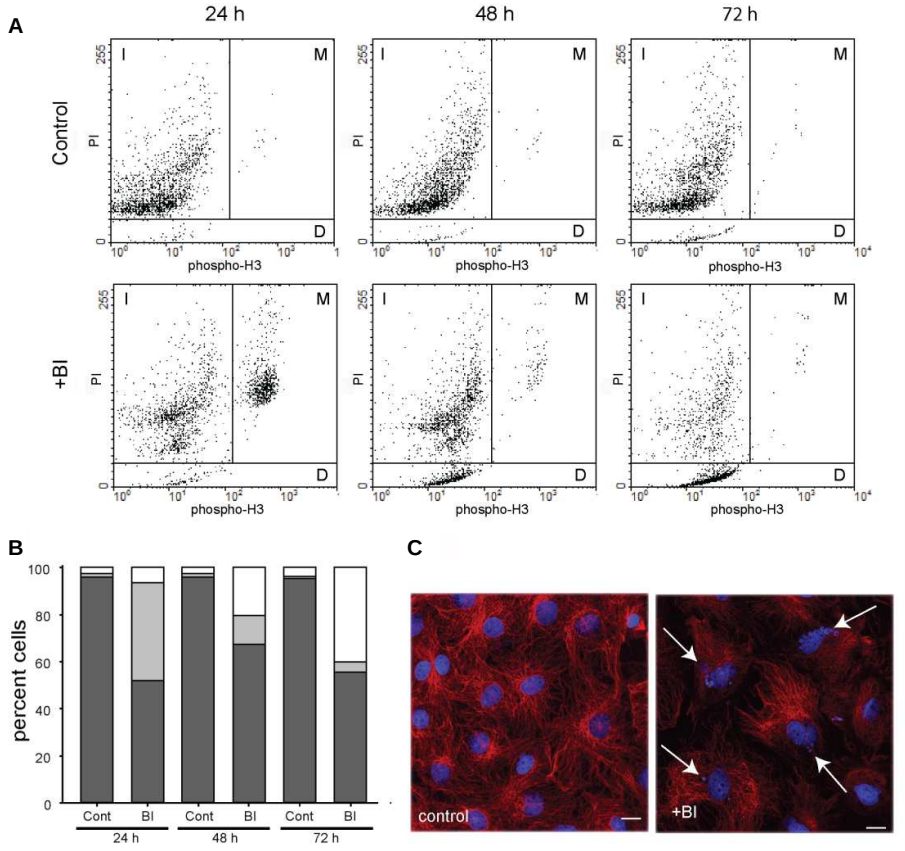
Chapter 3-Figure 1, page 46



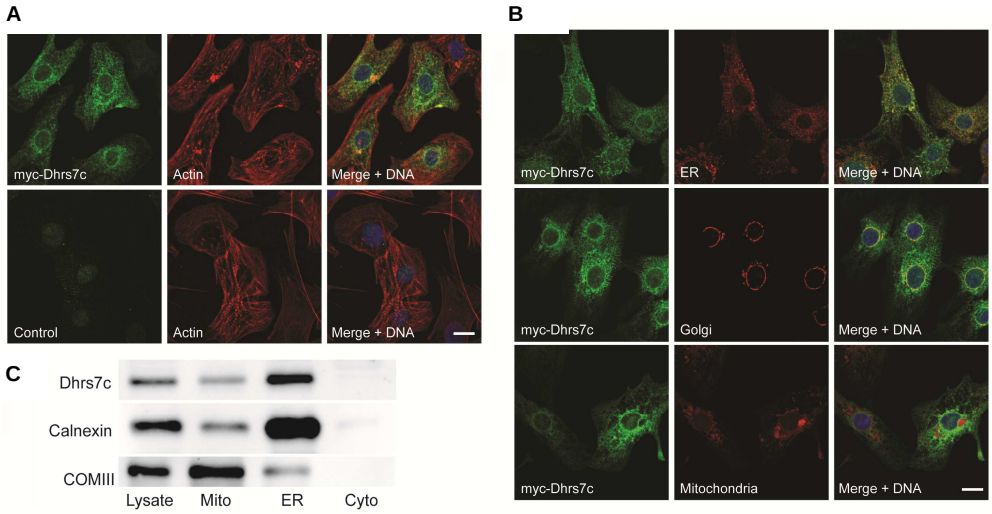
Chapter 3-Figure 4, page 50



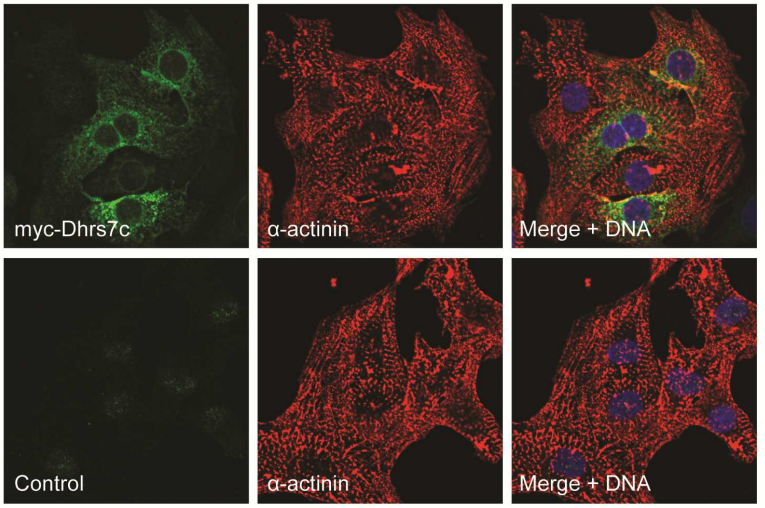
Chapter 3-Figure 5, page 51



Chapter 5-Figure 1, 91



Chapter 5-SFigure 2, page 105



Chapter 6-Figure 3, page 116